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Nitrogen Fixation in Beggiatoa, Vitreoscilla, and Thiothrix.

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NITROGEN FIXATION IN *BEGGIATOA*, *VITREOSCILLA*, AND *THIOTHRIX*

A Dissertation

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by
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ABSTRACT

Many strains of Beggiatoa, Vitreoscilla, and Thiothrix were found to be capable of nitrogen fixation and to harbor plasmid DNA of various sizes, including large plasmids, and several plasmids in the 12 to 15 Mdal size range.

Nitrogenase induction and activity in Beggiatoa alba were studied with respect to the effects of oxygen, pH, temperature, and nitrogenous compounds. Induction required microaerobic conditions. Nitrate and nitrite repressed induction, but glutamine did not. A pH optimum of 6.5 to 8.0 and a temperature optimum of 29°C were observed. Nitrogenase activity was immediately and incompletely inhibited by ammonium, but was not inhibited by nitrate, nitrite, and amino acids. The effects of different concentrations of methionine sulfoximine, an inhibitor of bacterial ammonium assimilation and transport, on ammonium inhibition of nitrogenase suggested that ammonium transport rather than assimilation was involved. Thallium inhibited activity similarly to ammonium, which, along with other experimental observations, implies that inhibition by ammonium is caused by the ionic effect that ammonium transport may have on the proton motive force.

An effort was made to clone *Beggiatoa alba nif* genes. Recombinant DNA molecules containing *B. alba* DNA and pBR322 were used to transform *E. coli*. However, the *Klebsiella pneumoniae nifHDK* probe that was to be used for screening recombinants showed weak hybridization with *B. alba* DNA. Unsuccessful attempts were made to optimize hybridization. During this portion of the investigation, it was found that *B. alba* B18LD DNA is resistant to digestion by several restriction endonucleases, which suggests that the DNA contains modified bases.

A technique was developed for purifying DNA from agarose gels. The procedure is an improvement over others because it is rapid, easily reproducible, and gives good yields of DNA.

Part I. Nitrogen fixation and plasmid DNA in *Beggiatoa*, *Vitreoscilla*, and *Thiothrix*.

INTRODUCTION

Beggiatoa are gliding, filamentous, sulfide-oxidizing bacteria (86). They have been found in such habitats as freshwater and coastal marine sediments (18, 33, 90), deep sea hydrothermal vent sites (29), and the rice plant rhizosphere (69). Environments in which Beggiatoa flourish are typically rich with sulfide (86). Beggiatoa are microaerophilic and grow at the interface of microoxic and anoxic zones of both laboratory simulations of their natural environment and chemically defined media (34, 64).

The types of nutrition that occur among the different species and strains of Beggiatoa include heterotrophy and mixotrophy in freshwater species (61, 62, 88, 91), as well as facultative chemoautotrophy in one marine strain (63). All are able to use acetate as a sole carbon and energy source, and mixotrophic and autotrophic Beggiatoa use reduced sulfur ions such as sulfide and thiosulfate as energy sources also (86). In mixotrophic Beggiatoa, sulfide oxidation and acetate oxidation are concurrent, oxygen-dependent, and appear to be linked to an electron transport chain which comprises a variety of cytochromes (79, 93).

Several strains of marine and freshwater Beggiatoa

are capable of nitrogen fixation as indicated by their ability to grow in the absence of any nitrogen source other than dinitrogen gas, and by their possession of the typical nitrogenase enzyme activity of reducing acetylene to ethylene (65). Nitrogenase is the abbreviated term for an enzyme complex which is found in nitrogen-fixing organisms and is composed of subunits of the enzymes dinitrogenase and dinitrogenase reductase (72). Nitrogenase catalyzes the reduction of N_2 to NH_3 (nitrogen fixation), and the NH_3 can then either be assimilated by the cell, or transported out of the cell and supplied to a symbiotic partner (24, 72). This catalysis is probably the most significant role of this enzyme in the physiology of a nitrogen-fixing organism. Nitrogenase also reduces other substrates such as H^+ and acetylene, C_2H_2 (24). The product of C_2H_2 reduction is ethylene, C_2H_4, which can be detected and quantified easily and rapidly by gas chromatography (71, 98). Acetylene reduction is frequently used to assay nitrogenase activity and no other enzyme has been found that possesses this activity (71, 98). The discovery of nitrogenase activity in Beggiatoa was fairly recent, in 1982 (65).

Beggiatoa are economically and ecologically important for many reasons. They are involved in

hydrogen sulfide detoxification in the rice rhizosphere (35), and they have been implicated in the bulking of activated sludge in waste water treatment plants (95, 102). They are also associated with the "black-line disease" of corals (13), and they are presumed to play major roles in the sulfur and carbon cycles in marine environments (33). Their ability to fix nitrogen suggests that they are important in the nitrogen cycle as well.

The genus Vitreoscilla contains non-sulfide-oxidizing, filamentous, gliding bacteria (87). Two of the species, V. beggiatoides and V. filiformis, are morphologically very similar to Beggiatoa, except that they lack the characteristic sulfur granules of Beggiatoa (92). Although these species are unable to oxidize sulfide, they are often maintained on media containing sulfide, and are found in the same type of sulfide-abundant environments where Beggiatoa also dwell (87, 92). V. beggiatoides and V. filiformis have many physiological characteristics in common with freshwater Beggiatoa. These include: heterotrophic growth on acetate, oxygen-dependent oxidation of acetate, mesophilic growth temperature range, incapability of anaerobic growth, ability to use NH_3, NO_2^-, and NO_3^- as sole nitrogen sources, lack

of catalase, and possession of the same types of cytochromes (87, 92).

Thiothrix are filamentous, sulfide-oxidizing bacteria that are similar to Beggiatoa in their ability to form sulfur granules through the physiological oxidation of sulfide, and in their possession of a mixotrophic nutrition (46). The only accepted species is T. nivea (46). The strains of this bacterium were isolated from sources of running water rich in sulfide (45), whereas Beggiatoa are typically isolated from stationary water sources (90). Like Beggiatoa, Thiothrix also thrives on acetate as a carbon and energy source (48). However, unlike Beggiatoa, T. nivea is an obligate mixotroph, and cannot grow on acetate, or any other carbon compound, in the absence of reduced sulfur ions (48, 91). T. nivea also differs from Beggiatoa and Vitreoscilla in that only the gonidia are capable of gliding motility; ensheathed Thiothrix filaments do not glide (46, 47).

Prior to this study, nitrogen-fixing ability had not been ascribed to either Vitreoscilla or Thiothrix. It was suspected that they might be nitrogen fixers because the low oxygen content of their natural environments is amenable to nitrogen fixation, a process which is usually extremely oxygen sensitive (72).

Plasmid DNA was recently detected in certain strains of Beggiatoa, Vitreoscilla, and Thiothrix using a method of plasmid isolation which allows the detection of small molecular weight plasmids (59). The results of that study are summarized in Table 1. No function has yet been assigned to any of these plasmids. Since nif (nitrogen fixation) genes are located on plasmid DNA in Rhizobium (3, 67, 81), the possibility exists that they are also located on plasmids in Beggiatoa, Vitreoscilla, and Thiothrix.

The research described in the first part of this dissertation was undertaken:

1. to determine the extent of nitrogen-fixing ability among several strains of Beggiatoa, Vitreoscilla, and Thiothrix,
2. to examine the plasmid DNA content in these organisms, and
3. to determine if a correlation exists between the presence of specific types of plasmids and the ability to fix nitrogen.

Table 1. Plasmid DNA in *Beggiatoa*, *Vitreoscilla*, and *Thiothrix*.*

Strain	Number of plasmid types	Sizes of plasmids (Mdal)
B. alba		
B18LD	2	12.3, 12.8
B15LD	3	12.1, 12.8, 13.2
B25RD	2	12.3, 12.8
Beggiatoa spp.		
PD1	0	---
V. filiformis		
L1401-2	1	5.5
ATCC 15551	0	---
V. beggiatoides		
B23SS	0	---
T. nivea		
JP3	1	8.1

* Data taken from Minges et al. (59).

MATERIALS AND METHODS

Maintenance of cultures. The strains of Beggiatoa, Vitreoscilla and Thiothrix that were used for this research are listed in Table 2. All strains of Beggiatoa and Vitreoscilla were maintained in the dark at 25°C, in plastic petri dishes containing modified MP05 medium plus 1.5% agar. Thiothrix were maintained in 25 ml glass, screw-top tubes, each containing 13 ml of modified MP05 medium plus 0.2% agar. The tube caps were slightly loose to allow the entry of air. All organisms mentioned above were maintained by transference into fresh media in duplicate every two weeks. 1 liter of modified MP05 medium is prepared by autoclaving separately three solutions which are combined after cooling down. The three solutions are:

1. 947 ml aqueous solution containing 3.7 mM NH_4Cl, 3.7 mM $CH_3CO_2Na \cdot 3H_2O$, 40 µM $MgSO_4 \cdot 7H_2O$, 20 ml saturated $CaSO_4 \cdot 2H_2O$, 100 µl of a solution containing 0.68 mM EDTA, pH 8.0, and 2.5 mM $FeSO_4 \cdot 7H_2O$, and 5 ml of a trace elements solution containing 35 µM $ZnSO_4 \cdot 7H_2O$, 120 µM $MnSO_4 \cdot H_2O$, 20 nM $CuSO_4 \cdot 5H_2O$, 26 µM $Na_2B_4O_7 \cdot 10H_2O$, 4.2 µM $CoCl_2 \cdot 6H_2O$, and 4 µM $Na_2MoO_4 \cdot 2H_2O$. If agar is to be included

Table 2. Strains of *Beggiatoa*, *Vitreoscilla*, and *Thiothrix* used in this study.

Strain	ATCC #	Source or reference
Beggiatoa alba		
B18LD	33555	(58)
B15LD	33554	(58)
B25RD	33556	(58)
Beggiatoa spp.		
f3	none	J. Larkin
f17	none	J. Larkin
R3	none	J. Larkin
R17	none	J. Larkin
IIIf14	none	J. Larkin
SM1	none	W. Strohl
B40CA	none	W. Strohl
B37FL	none	W. Strohl
OH-75-B-cl2a	none	(61)
L1401-15	none	(58)
PD1	none	(93)

Table 2 cont'd.

Strain	ATCC #	Source or reference
V. filiformis		
ATCC 15551	15551	(92)
L1401-2	43190	(92)
L1401-6	43191	(92)
V. beggiatoides		
B23SS	43189	(92)
T. nivea		
JP1	35099	(48)
JP2	35100	(48)
Thiothrix spp.		
A	none	J. Larkin
7	none	J. Larkin
JP8	none	J. Larkin

in the medium, then it should added to this solution before autoclaving.

2. 50 ml 50 mM KH_2PO_4, pH 7.0.
3. 3 ml 10% (w/v) aqueous $Na_2S \cdot 9H_2O$.

Most of the composition of modified MP05 medium is derived from the original MP05 described previously (88, 90).

Screening for nitrogen-fixing potential.

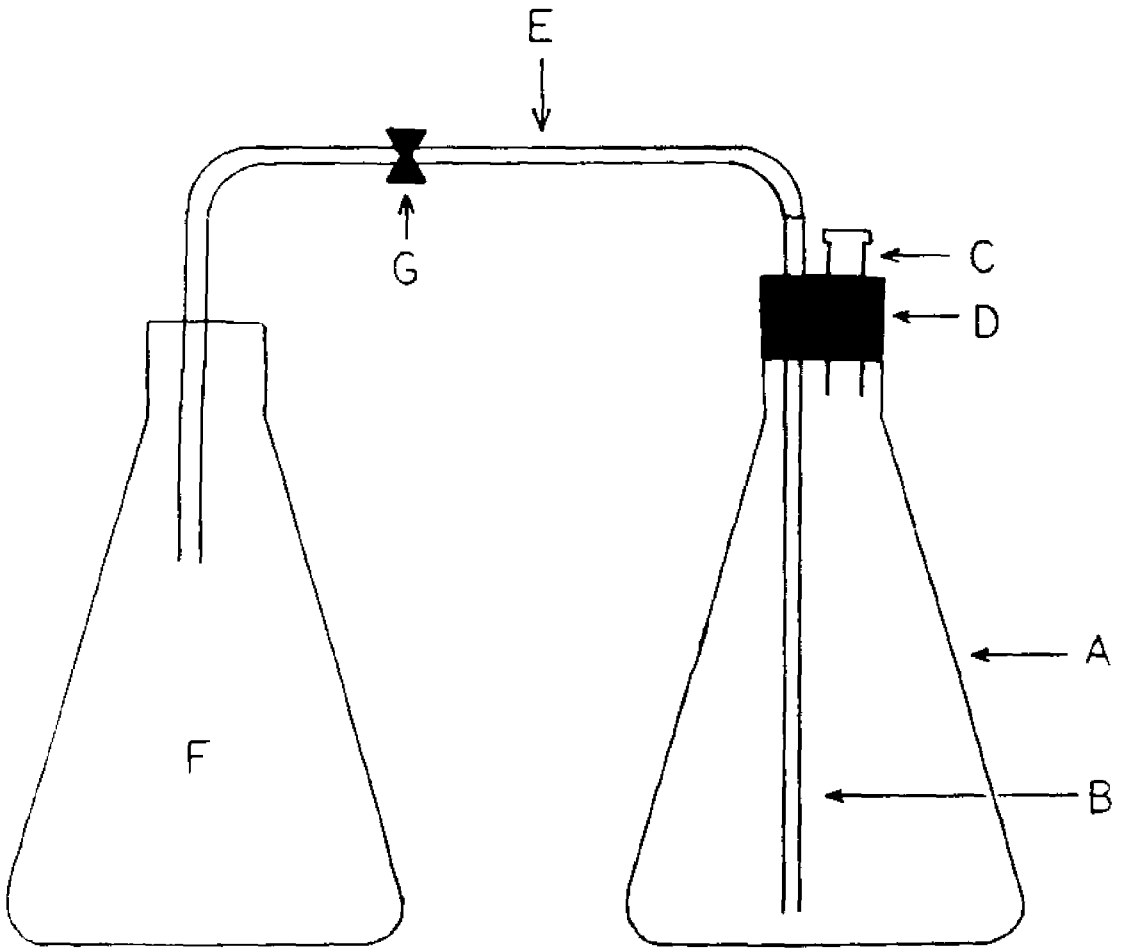
Small aliquots of bacteria were transferred from the maintenance media to 20 ml test tubes containing 10 ml nitrogen-deficient modified MP05 medium (NDMP) plus 0.2% Noble agar. NDMP differed from the modified MP described above in that the NH_4Cl had been excluded, and the final concentrations of $MgSO_4 \cdot 7H_2O$ and $Na_2MoO_4 \cdot 2H_2O$ were raised to 0.8 mM and 1 μM, respectively (83). For Beggiatoa and Vitreoscilla strains, a small aliquot of cells consisted of a circular piece of agar 3 mm in diameter and 1 mm in depth from a maintenance culture. A small aliquot of Thiothrix consisted of 100 μl of cell suspension from tubes containing maintenance media. In either case cells were suspended in the upper 10 mm of the semi-solid NDMP medium. Foam plugs were inserted into the openings of the tubes, and the tubes were placed into a plastic evacuation chamber. The chamber was evacuated with a vacuum pump until the vacuum gauge on

the pump read 24 inches Hg. The vacuum within the chamber was replaced with pure nitrogen gas. Thus, the final composition of the atmosphere within the chamber was 95.6% N_2, 4.2% O_2, and 0.2% minor atmospheric gases. This composition was inexactly derived from atmospheric conditions previously described for growing Beggiatoa diazotrophically (65). The chamber was sealed and incubated in the dark at 25°C. 7 to 14 days later, when bacterial growth was visible in the upper 10 mm of the medium, the cultures were removed from the chamber, and 100 µl portions of cell suspension from each culture were transferred to the upper 10 mm of medium in 30 ml serum tubes containing 15 ml of the same type of medium. Foam plugs were inserted into the serum tube openings and the tubes were incubated in an evacuation chamber as described above.

Approximately one week after the inoculation of the serum tubes, bacterial growth was discernible in each tube. The evacuation chamber was opened and the tubes were quickly sealed with serum caps. The closed tubes were incubated overnight in the dark at 25°C, after which 1.5 ml of the tube headspace was removed with a 3 ml syringe and 1.5 ml of acetylene was injected immediately into the tube with the same syringe. Acetylene was produced in the lab by adding calcium

carbide to deionized water (71) in the device shown in Figure 1. The entire volume of flask A, except for 2 ml, was initially completely occupied by distilled water. 1.8 g of calcium carbide was quickly added into the serum tube opening (C) with a scoop-type spatula (Sargent-Welch S-75290) and the opening was immediately sealed with a serum cap. Acetylene production began immediately and, after 200 to 300 ml of the distilled water had been displaced by acetylene into the waste collection flask (F), the rubber tube leading into flask F was clamped shut (G). Samples of acetylene were removed by syringe from the serum cap and injected directly into the serum tubes containing the bacterial cultures. The culture tubes were incubated overnight in the dark at 25°C, and the production of ethylene was measured by removing 500 µl headspace samples with a gas-tight syringe, injecting them into a Perkins-Elmer 3920B gas chromatograph equipped with a Porapak N column, and recording ethylene peaks on a chart recorder. A standard curve was prepared by injecting known quantities of ethylene ranging from 1 to 15 nmoles in volumes of 50 to 500 µl into the gas chromatograph. The resultant ethylene peak heights were plotted against nmoles of ethylene present in the injected sample (71). The total amount of ethylene present in the headspace of each culture tube was

Figure 1. Device used for the production of acetylene. A, 500 ml flask; B, glass tubing; C, truncated serum tube; D, rubber stopper; E, rubber tubing; F, 500 ml flask; G, clamp.


E
G
C
D
A
B
F

determined from this curve.

After quantitation of ethylene, the upper 2 ml of each culture, which contained all visible traces of growth, was transferred to 1.5 ml microfuge tubes and centrifuged for 10 minutes in a microcentrifuge. This produced a pellet containing the cells and the agar. The clear supernatant was discarded and 10% (w/v) trichloroacetic acid was added to the pellet to bring the volume to 1 ml. The mixture was heated at 90°C for 20 minutes. This causes hydrolysis of the agar and precipitation of cell protein (65, 75, 84). The protein precipitate was pelleted by centrifugation in a microfuge for 5 minutes. The supernatant was discarded and the pellet was dissolved by adding 250 µl 0.1 N NaOH and incubating for 1 hour at 55°C (65). Total protein for each culture tube was determined by a previously described method (53) in the following manner. The protein solutions for a particular culture tube were combined and the volume was brought to 1 ml with distilled water. This was transferred to a 20 ml test tube and was well mixed with 5 ml of a freshly prepared solution made by dissolving 20 mg sodium tartrate, 10 mg $CuSO_4 \cdot 5H_2O$, 2 g of sodium carbonate, and 10 ml 1 N NaOH, in 90 ml distilled water, in that order. The protein mixture was then allowed to stand for 10 minutes

at room temperature. 500 µl of concentrated Folin-Ciocalteau reagent (Sigma Chemical Co.) was added, and the mixture was vortexed immediately for 5 seconds. After 1 hour the absorbance of the solution at 500 nm was measured with a spectrophotometer. A series of standards ranging from 20 to 400 µg protein, made with bovine serum albumin, was analyzed by the same method, and a standard curve was prepared from which the total protein of each culture was determined. In vivo nitrogenase rates were then calculated for each culture in the form nmoles C_2H_4/mg protein/minute. Duplicate or triplicate nitrogenase assays were used to derive an average enzyme rate for a particular bacterial strain. Identical assays were also done with 3.7 mM NH_4Cl present in the medium in order to demonstrate the absence of nitrogenase activity when this source of nitrogen was present. Negative controls consisting of uninoculated tubes of medium were done. These exhibited no ethylene production.

Plasmid isolation. Three different methods of plasmid isolation were used: the cleared-lysate method (22, 57, 59), the in-well lysis method (14, 77), and the Kado-Liu method (36).

A. Cleared-lysate method. A modificaton of previously reported methods (22, 57, 59) was used which

employed gentle mixing of solutions and the use of wide-bore pipette tips for the transfer of solutions containing DNA. These precautions were taken to minimize the shearing of large plasmid DNA molecules. 50 to 100 ml of a log phase cell suspension of Beggiatoa was added to 500 ml of modified MP05 broth in a 1 liter flask and this was shaken at 150 rpm, 29°C, for 16 to 24 hours (88). The cell suspension was centrifuged at 8000 x g for 10 minutes and the pellets were resuspended in 8 ml 10 mM Tris, pH 8.0, 1 mM EDTA (TE buffer). 16 mg of lysozyme dissolved in 500 µl of TE buffer was added to the cell suspension, which was then incubated at 37°C for 15 minutes. 100 mg of SDS was gently mixed into the suspension, followed by incubation at 60°C for 15 minutes. 3.3 ml of 4 M NaCl was added and the mixture was incubated at 4°C overnight. The lysate was centrifuged at 20,000 x g 30 minutes, and the supernatant was carefully removed with a wide-bore pipette, created by cutting off the narrow end of a 1000 µl plastic pipette tip (diameter of the orifice was 3 mm), and transferred to a 30 ml glass centrifuge tube. 18 ml of chloroform-isoamyl alcohol, 24:1 (v/v), was added to this solution, which was then gently mixed by inverting the tube several times over a 2 minute time period. The mixture was centrifuged at 12,000 x g for 15 minutes. 8

ml of the upper aqueous layer was removed with a wide-bore pipette and transferred to a 30 ml centrifuge tube. 0.8 ml of 3 M sodium acetate was added, mixed in, and then 17.6 ml of cold 95% ethanol was added and mixed in, followed by incubation at -20°C overnight. The tube was centrifuged at 12,000 x g 10 minutes, the supernatant was discarded, and the DNA pellet was allowed to air dry and then was gently dissolved in 0.5 to 1 ml TE buffer. The DNA solution was stored at 4°C in a 1.5 ml microfuge tube. A few days later 35 µl aliquots of the DNA solutions were mixed with 7 µl of gel loading buffer, which contained 0.25% (w/v) bromphenol blue and 40% (w/v) sucrose in water. The samples were loaded onto a horizontal agarose gel electrophoresis unit with a 0.7 % agarose slab (w/v in TBE buffer, pH 8.2) 20 cm long. TBE buffer contains, per liter: 10.9 g Tris base, 5.5 g boric acid, and 4 ml 0.5 M EDTA, pH 8.0 (55). The DNA samples were electrophoresed at 25 V for 36 hrs. After electrophoresis, the gel slab was stained in 500 ml of an aqueous ethidium bromide solution (0.5 µg/ml) for 30 minutes and then washed for 30 minutes in 500 ml deionized water (55). The stained gel slab was then exposed to long wavelength ultraviolet light with a transilluminator and a photograph was taken with Polaroid 665 film through an orange filter (55).

B. In-well lysis method. A modification of previously described in-well lysis methods was used (14, 77). 0.5 - 1.0 ml of a log phase Beggiatoa culture which contained 30 to 50 µg cell protein/ml was centrifuged for 2 min in a microfuge, resuspended in 500 µl TE buffer, centrifuged again, and then resuspended in 40 µl of a solution containing 0.2 mg/ml lysozyme, 3 µg/ml RNase A, 0.5 mg/ml bromphenol blue, and 560 mg/ml sucrose in TBE buffer, pH 8.2. The mixture was immediately loaded into the well of a 0.7% (w/v) vertical agarose gel slab made with TBE buffer (length, 14 mm; height, 17 mm; well dimensions, 12x8x2.5 mm). The entire electrophoresis chamber was then incubated at 37°C for 15 minutes. 40 µl of a solution containing 0.02 g/ml SDS (sodium dodecyl sulfate) and 280 mg/ml sucrose in TBE buffer was carefully layered over the lysozyme-treated cell suspension. 100 µl of a solution containing 0.02 g/ml SDS and 140 mg/ml sucrose in TBE was then added to the well. Electrophoresis was begun immediately and performed at 10 mA for 1 hour and then 40 mA for 11 hrs. The gel slab was stained with an ethidium bromide solution for 15 minutes, washed in deionized water for 10 minutes, and photographed while exposed to UV light, as described above.

C. Kado-Liu method of plasmid isolation (36). Beggiatoa, Vitreoscilla, and Thiothrix were grown to log phase in modified MP05 broth as described above for the cleared-lysate method except that Vitreoscilla and Thiothrix were shaken at 50 rpm for 2 to 3 days. 500 µl to 1 ml of a log phase culture was centrifuged for 5 minutes in a microfuge. The pellet was resuspended in 100 µl of TE buffer. 200 µl of a solution containing 3% SDS in 50 mM Tris, pH 12.5, was added and mixed into the cell suspension by gently inverting the tube twice. The mixture was incubated at 55°C for 1 hour. 600 µl of phenol-chloroform, 1:1 (v/v), was added and mixed in by gentle inversion. The tube was centrifuged 5 minutes. 35 to 40 µl samples of the aqueous upper layer were transferred to microfuge tubes, mixed with 7 to 8 µl of gel loading buffer, and electrophoresed as described above for the cleared-lysate method, except that electrophoresis was performed at 100 V for 4 to 8 hours. Gel slabs were stained for 30 minutes, washed, and photographed as mentioned above.

RESULTS AND DISCUSSION

Of all the strains tested, only *Beggiatoa* sp. PD1 and *Thiothrix* sp. JP8 did not reduce acetylene to ethylene (Table 3). All strains except PD1 grew in nitrogen-deficient medium (Table 3). Strain PD1 may not be a member of *Beggiatoa* since it is inconsistent in its ability to oxidize sulfide (93). The observation that JP8 grew in NDMP, yet did not show ethylene production, may be due to undetectable levels of acetylene reduction activity. For all but one of the strains capable of reducing acetylene, it was demonstrated that the presence of NH_4Cl in the assay medium inhibited the production of ethylene (Table 3). This establishes the acceptibility of using the acetylene reduction assay for analyzing the nitrogen fixing metabolism of these strains. The results shown in Table 3 demonstrate that the ability to fix nitrogen is widespread among members of the genus *Beggiatoa*. Since nearly all of the *Beggiatoa*, *Vitreoscilla*, and *Thiothrix* strains tested are nitrogen fixers, diazotrophy may be a general feature of bacteria which are commonly found in sulfide-enriched environments.

Specific nitrogenase activities for some *Beggiatoa* strains are recorded in Table 4. The values for average

Table 3. Growth in NDMP and ethylene production by *Beggiatoa*, *Vitreoscilla*, and *Thiothrix*

Strain	Growth in NDMP	C_2H_4 production in absence of NH_4Cl	C_2H_4 production in presence of NH_4Cl
B. alba			
B18LD	+	+	-
B15LD	+	+	-
B25RD	+	+	-
Beggiatoa spp.			
f3	+	+	-
f17	+	+	-
R3	+	+	-
R17	+	+	-
IIIf14	+	+	-
SM1	+	+	-
B40CA	+	+	-
B37FL	+	+	-
OH-75-c12a	+	+	-
L1401-15	+	+	-
PD1	-	-	n.d.

Table 3 cont'd.

Strain	Growth in NDMP	C_2H_4 production in absence of NH_4Cl	C_2H_4 production in presence of NH_4Cl
V. filiformis			
ATCC 15551	+	+	-
L1401-2	+	+	-
L1401-6	+	+	-
V. beggiatoides			
B23SS	+	+	-
T. nivea			
JP1	+	+	-
JP2	+	+	-
Thiothrix spp.			
A	+	+	-
7	+	+	n.d.
JP8	+	-	n.d.

Table 4. Nitrogenase specific activities and amount of protein for Beggiatoa grown in semi-solid NDMP.

Strain	Total µg protein in culture tube	Sp. activity for culture tube[a]	Mean activity +/- S.D.[b]
B18LD	342.4	0.436	
	261.2	0.483	
	162.4	0.903	0.61 +/- 0.26
B15LD	305.0	0.543	
	177.6	1.530	
	117.6	2.700	1.59 +/- 1.08
B25RD	337.6	0.370	
	322.4	0.402	
	267.6	0.429	0.40 +/- 0.03
f3	240.0	0.650	
	258.8	0.679	
	232.4	0.740	0.69 +/- 0.04
f17	207.6	0.826	
	105.0	1.610	
	92.4	1.890	1.44 +/- 0.55

Table 4 cont'd.

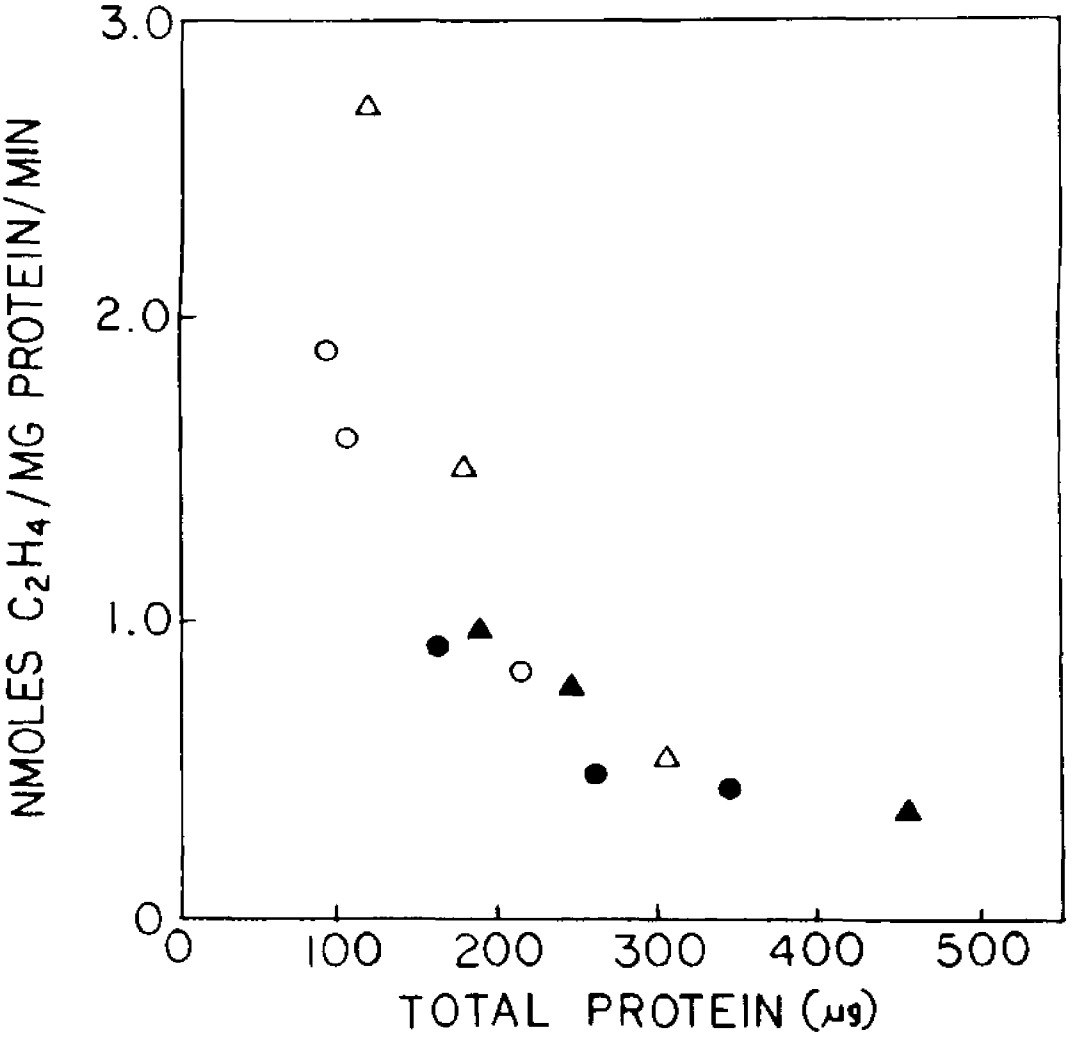
Strain	Total μg protein in culture tube	Sp. activity for culture tube[a]	Mean activity +/- S.D.[b]
R17	150.0	1.429	
	42.4	4.780	3.10 +/- 2.37
R3	327.6	0.528	
	170.0	0.899	0.71 +/- 0.26
SM1	192.4	0.596	
	190.0	0.741	
	152.4	1.204	0.85 +/- 0.32
B37FL	120.0	0.770	
	127.6	1.130	0.95 +/- 0.18
OH-75-c12a	455.0	0.361	
	245.0	0.761	
	188.8	0.948	0.69 +/- 0.30

[a] Nanomoles C_2H_4 produced/mg protein/minute.

[b] S.D., standard deviation.

activities range from 0.40 to 3.10 nmol C_2H_4/mg protein/minute. Some of these activities have high standard deviations that are indicative of the variability of the individual enzyme rates from which these averages were derived (See data for B18LD, B15LD, f17, R3, R17, and OH-75-c12a in Table 4). In these cases, as shown in Table 4 and in Figure 2, there is an inverse relationship between the amount of protein in the cultures and the enzyme specific activity. Figure 2 illustrates the consistency of this relationship among the values recorded for some of the strains. For the average enzyme rates which show very low standard deviations, the protein values are much less variable (See B25RD, f3 in Table 4). The inverse relationship between culture protein and nitrogenase activity is also exemplified by the observation that the lowest amount of protein in any individual culture tube was 42.4 μg for strain R17, and this culture showed the highest enzyme rate, 4.78, and the highest amount of protein in any individual culture was 455.0 μg for strain OH-75-c12a, and this culture showed the lowest enzyme rate, 0.361 (Table 4). There are two possible reasons for the inverse relationship between total protein and specific activity. One possibility is that the method of extracting protein was less efficient at lower protein

Figure 2. Beggiatoa nitrogenase specific activities plotted against total protein for individual assay cultures. The symbols represent values for the following strains: B15LD (△), f17 (○), OH-75-c12a (▲), B18LD (●).


3.0
2.0
1.0
0
0
100
200
300
400
500
NMOLES C_2H_4/MG PROTEIN/MIN
TOTAL PROTEIN (μg)

concentrations. Another likely possibility is that cultures which had higher amounts of protein had grown to the extent that one or more nutrients in the semi-solid medium had become limiting to growth and respiration, and the cells were thus less capable of fixing nitrogen and reducing acetylene. The variability in the amount of growth in each culture, as indicated by the protein content, was probably due to the difficulty in transferring replicate quantities of cells from culture tube to culture tube, since none of the strains listed in Table 4 grow evenly dispersed within the tubes. Instead, growth occurs in narrow discs 1 to 2 mm in thickness that lie a few mm below, and parallel to, the surface of the medium. In conclusion, the tubes of semi-solid medium are excellent for detecting the ability to fix nitrogen. However, when using this method of cultivation in the determination of specific enzyme activities, care should be taken to inoculate and monitor the cultures so that growth has not become limited at the time of the nitrogenase assay. When this precaution is taken, the specific activities will more accurately reflect the full potential of a particular strain for nitrogen fixation.

As shown in Table 5 and Figures 3 through 6, a variety of plasmids was found in the bacterial strains tested. There is a predominance of plasmid DNA in the 12

Table 5. Plasmid DNA in *Beggiatoa*, *Vitreoscilla*, and *Thiothrix*.

Strain	Number of plasmid types	Sizes of plasmids (Mdal)	Method(s) used for detection[a]
B. alba			
B18LD	3	12.3, 12.8, LP[c]	CL, IW, KL
B15LD	3	12.1, 12.8, 13.2	CL, KL
B25RD	2	12.3, 12.8	CL, KL
Beggiatoa spp.			
f3	2	13.0 +/- 0.2[b], LP	CL, IW, KL
f17	2	13.2 +/- 0.3[b], LP	CL, KL
R3	2	13.2 +/- 0.0[b], LP	CL, KL
R17	1	LP	CL, IW, KL
IIIf14	2	15.0 +/- 0.1[b], LP	CL, KL
OH-75-c12a	1	13.0	KL
SM1	2	14.0, LP	KL
B40CA	0	---	KL
L1401-15	1	13.6	KL
PD1	2	LP, LP	KL
B37FL	0	---	KL

Table 5 cont'd.

Strain	Number of plasmid types	Sizes of plasmids (Mdal)	Method(s) used for detection[a]
V. filiformis			
L1401-6	1	6.8	KL
V. beggiatoides			
B23SS	0	---	KL
T. nivea			
JP1	1	LP	KL
JP2	1	LP	KL
Thiothrix spp.			
A	0	---	KL
7	0	---	KL
JP8	0	---	KL

[a] CL, cleared lysate; IW, in-well lysis; KL, Kado-Liu.

[b] Average of 2 separate size determinations from different electrophoretic runs.

[c] LP, large plasmid of undetermined size.

Figure 3. Analysis of plasmid DNA in Beggiatoa by the cleared lysate method. The strain that was tested and the identity of DNA bands in each lane from top to bottom are: lane 1, strain R3 - large plasmid, chromosomal DNA, 13.2 Mdal plasmid; lane 2, strain R17 - large plasmid, chromosomal DNA; lane 3, strain B25RD - chromosomal DNA, 12.8 Mdal plasmid, 12.3 Mdal plasmid; lane 4, strain B15LD - chromosomal DNA, 13.1 Mdal plasmid, 12.8 Mdal plasmid, 12.1 Mdal plasmid; lane 5, strain B18LD - chromosomal DNA, 12.8 Mdal plasmid, 12.3 Mdal plasmid; lane 6, strain f17 - large plasmid, chromosomal DNA, 13.2 Mdal plasmid; lane 7, strain IIIf14 - chromosomal DNA, 15.0 Mdal plasmid; lane 8, strain f3 - chromosomal DNA, 13.0 Mdal plasmid. The symbol "o" refers to the position of the gel origin and the symbol "c" refers to the position of the chromosomal DNA.

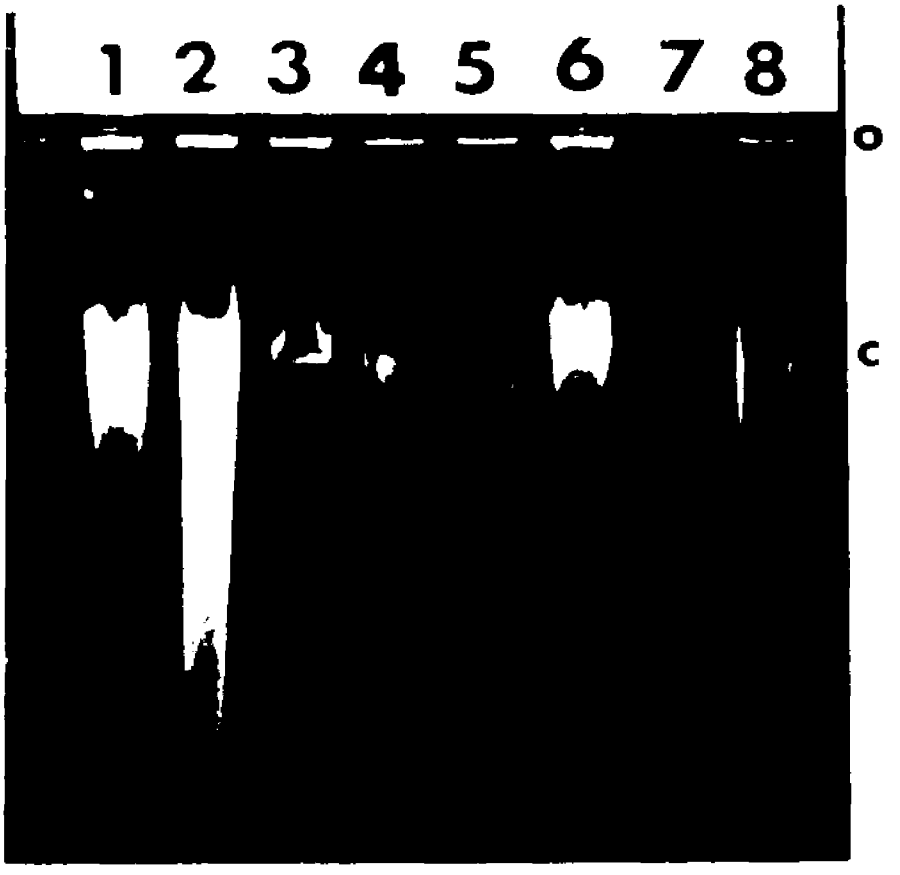

1 2 3 4 5 6 7 8
o
c

Figure 4. Analysis of plasmid DNA in Beggiatoa by the in-well lysis method. The strain that was tested and the identity of DNA bands in each lane from top to bottom are: lane 1, strain f3 - large plasmid, chromosomal DNA, 13.0 Mdal plasmid; lane 2, strain R17 - large plasmid, chromosomal DNA. The symbol "o" refers to the position of the gel origin and the symbol c"" refers to the position of chromosomal DNA.


1
2
o
c

Figure 5. Analysis of plasmid DNA in Beggiatoa and Thiothrix by the Kado-Liu method. The strain that was tested and the identity of DNA bands in each lane from top to bottom are: lane 1, strain f3 - 13.0 Mdal plasmid; lane 2, strain OH-75-c12a - 13.0 Mdal plasmid; lane 3, strain f17 - 13.2 Mdal plasmid; lane 4, strain B15LD - 13.1 Mdal plasmid, 12.8 Mdal plasmid, 12.1 Mdal plasmid; lane 5, strain B18LD - 12.8 Mdal plasmid, 12.3 Mdal plasmid; lane 6, strain f3 - 13.2 Mdal plasmid; lane 7, strain JP2 - large plasmid; lane 8, strain R17 - no bands; lane 9, strain B25RD - 12.8 Mdal plasmid, 12.3 Mdal plasmid; lane 10, strain IIIf14 - large plasmid, 15.0 Mdal plasmid; lane 11, strain SM1 - large plasmid, 14.0 Mdal plasmid; lane 12, strain JP1 - large plasmid. The symbol "o" refers to the position of the gel origin. The large plasmid band for strain IIIf14 in lane 10 is very faint and comigrates with the large plasmid band for strain SM1 in lane 11.

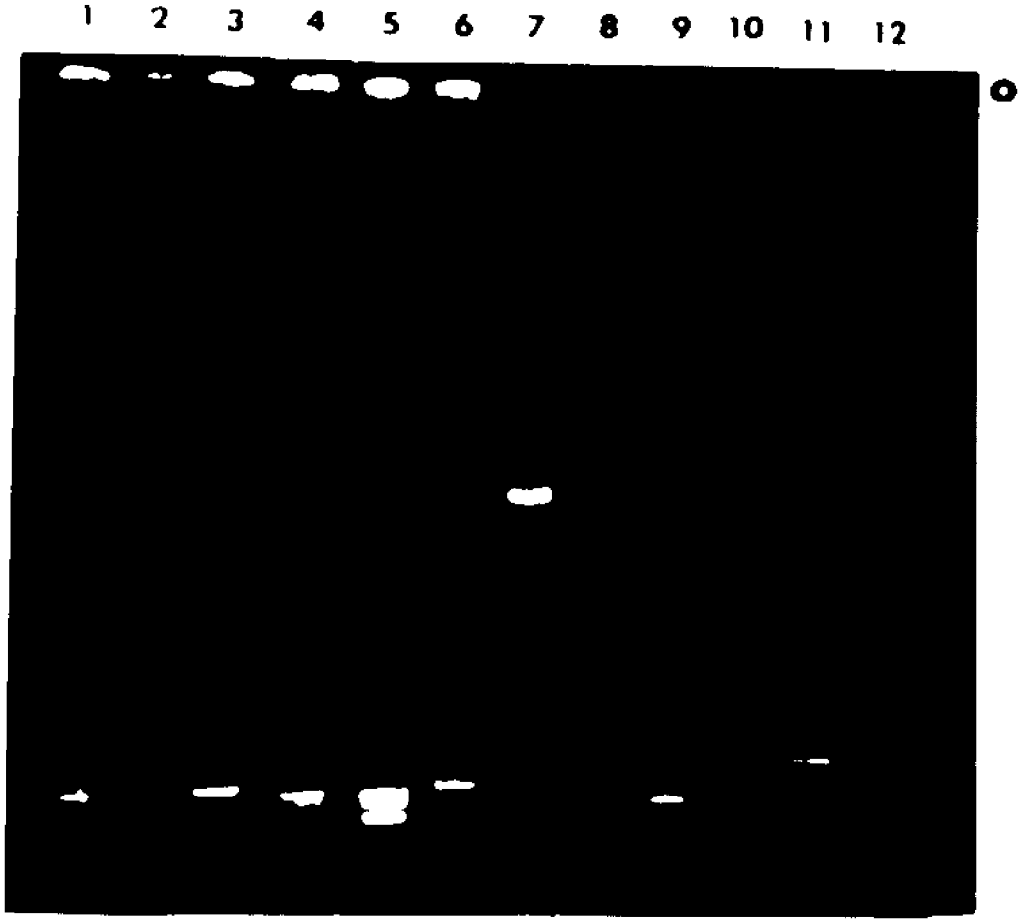
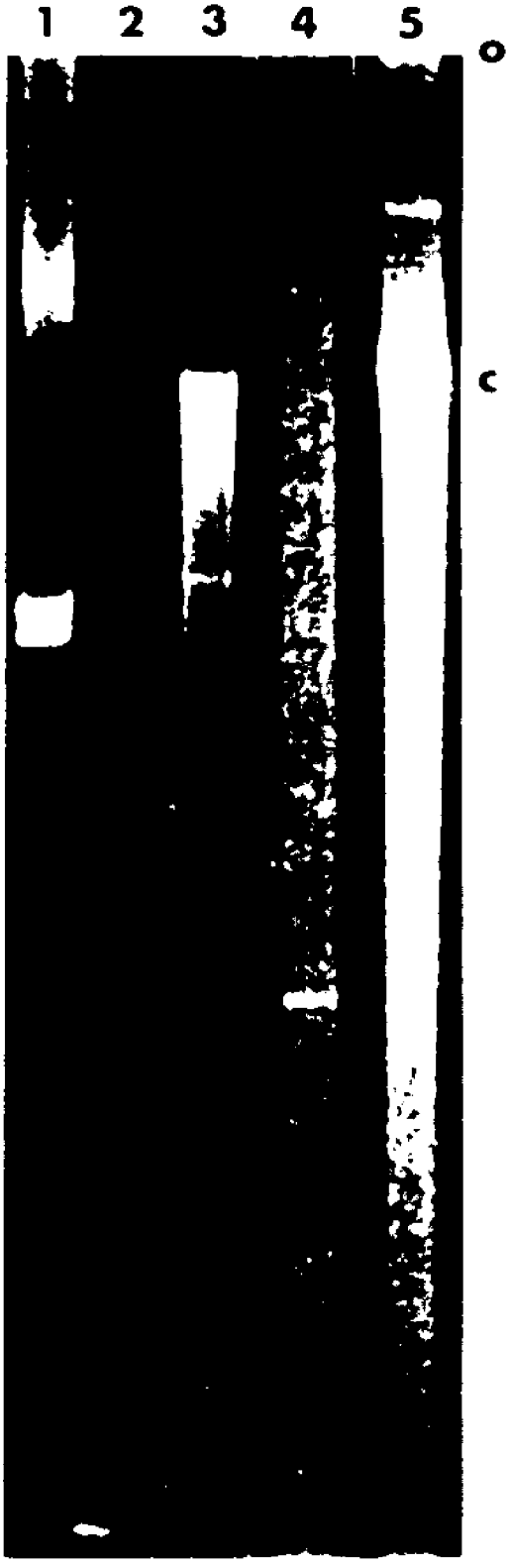

1
2
3
4
5
6
7
8
9
10
11
12
O

Figure 6. Analysis of plasmid DNA in Beggiatoa and Vitreoscilla by the Kado-Liu method. The strain that was tested and the identity of DNA bands in each lane from top to bottom are: lane 1, strain B18LD - chromosomal DNA, large plasmid, 12.8 Mdal plasmid, 12.3 Mdal plasmid; lane 2, plasmid pBR322 size standards - open circular form of 5.8 Mdal dimer, open circular form of 2.9 Mdal monomer, closed circular form of 5.8 Mdal dimer, closed circular form of 2.9 Mdal monomer; lane 3, strain L1401-15 - chromosomal DNA, 13.6 Mdal plasmid; lane 4, strain L1401-6 - chromosomal DNA, 6.8 Mdal plasmid; lane 5, strain PD1 - large plasmid, large plasmid, chromosomal DNA. The symbol "o" refers to the position of the gel origin and the symbol "c" refers to the position of chromosomal DNA.


1
2
3
4
5
o
c

to 15 Mdal size range in the Beggiatoa strains (Table 5). No plasmids smaller than 12 Mdal were found in any of the Beggiatoa strains. Large plasmids of undetermined size, which formed electrophoresis bands that, in most cases, migrated above the smear of chromosomal DNA, were found in 8 of the Beggiatoa strains and both of the T. nivea strains (Table 5, Figures 3, 4, 5, and 6).

Of the three methods used for plasmid isolation, the Kado-Liu method was the easiest to perform and the best for producing nearly pure preparations of the smaller sized plasmids (Figure 5). However this method was not adequate for detecting the larger plasmids found in strains f3, f17, R3, and R17, whereas the cleared lysate and in-well lysis methods were adequate for this purpose (compare Figures 3 and 4 with Figure 5). The Kado-Liu method was adequate for detecting large plasmids in strains B18LD, SM1, PD1, IIIf14, JP1, and JP2 (Figures 5 and 6). The large plasmids in these strains may be smaller than those found in f3, f17, R3, and R17, which might explain why the Kado-Liu method did not allow the detection of all of the large plasmids.

Since most of the bacterial strains contain plasmid DNA and all but 2 strains are nitrogen fixers, a good correlation between a nitrogen fixation phenotype and the

presence of plasmid DNA is not possible. However, some weak correlations can be presented. The observation that all three nitrogen-fixing B. alba strains contain 12.8 Mdal plasmids indicates that these plasmids could contain nif genes. Nitrogen-fixing Beggiatoa sp. strains f3, f17, R3, and R17 are morphologically similar strains which were all isolated from Louisiana rice fields, and probably are members of the same species. Since strain R17 does not carry a 13 Mdal plasmid like the other strains of this group, this probably excludes the possiblity of nif genes residing on the 13 Mdal plasmids. However, these strains all contain large plasmids which, at least in the case of R3 and R17, appear to migrate at approximately the same rate on agarose gels (Figure 3). These large plasmids might contain nif genes, especially since nif genes are located in very large plasmid DNA molecules in more than one Rhizobium species (3, 67, 81). Among the other plasmid types shown in Table 5, the only other possible correlation between plasmid DNA and the ability to fix nitrogen is the presence of comigrating large plasmids in both of the nitrogen-fixing T. nivea strains. All of the proposed correlations mentioned above are indefensible, since in no instance does the absence of a nitrogen fixation phenotype coincide with the absence of

a particular type of plasmid DNA. Because no good correlation was made, it is more likely that the ability to fix nitrogen is chromosomally-encoded in these organisms.

The predominance of 12 to 15 Mdal plasmids in Beggiatoa that is indicated by this study suggests that a conserved size range for plasmid DNA occurs in these bacteria. Since the plasmids are of unknown function, no selective advantage for their presence in the cells is known. They may represent forms of bacteriophage DNA. Bacteriophage-like particles have recently been observed in electron micrographs of Beggiatoa (J. M. Larkin and M. C. Henk, unpublished observations), and plaques occasionally occur on aged plate cultures of strain B18LD (J. K. Polman, unpublished observations). The large plasmids which occur in T. nivea may be conjugative plasmids, since fimbriae which are associated with cell-to-cell contact have been observed in T. nivea (47).

Part II. Physiological properties of in vivo nitrogenase activity in *Beggiatoa alba* B18LD.

INTRODUCTION

Beggiatoa alba strain B18LD was originally isolated from freshwater sediments in south Louisiana (90). It was designated as the type strain for the species in 1984 (58).

B. alba B18LD is considered to have a mixotrophic nutrition because it can use inorganic and organic energy sources concurrently (21, 91). The organism can use sulfide or thiosulfate as inorganic energy sources and acetate and other carbon compounds as carbon and energy sources (21, 88, 91). *B. alba* B18LD is a facultative mixotroph in that it can also grow heterotrophically on acetate and other carbon sources in the absence of reduced sulfur compounds (88). It has been demonstrated with continuous cultures of *B. alba* B18LD that sulfide increases the efficiency of growth on acetate (21).

B. alba B18LD oxidizes sulfide to elemental sulfur, which is deposited externally as granules that lie within invaginations of the cytoplasmic membrane (49, 50, 80, 89). The elemental sulfur is not further oxidized to sulfate (79) as it is in a marine chemoautotrophic *Beggiatoa* (63). The sulfur can be reduced back to sulfide by *B. alba* B18LD under

anaerobic conditions (79). This reduction accompanies the activity of an uptake hydrogenase which oxidizes hydrogen gas while elemental sulfur is being reduced to sulfide (79). Under these circumstances the sulfur is thought to be an alternate electron acceptor to oxygen, but the process appears to be capable of providing only enough energy for maintenance, not for growth (79).

Both sulfide oxidation to sulfur and acetate oxidation to carbon dioxide are linked to oxygen consumption by a respiratory chain, and the oxygen-dependent oxidation of either ion (sulfide or acetate) partially inhibits the oxidation of the other ion (8, 79, 93). The following electron transport components have been discovered in B. alba B18LD, and some or all may be involved in an energy-producing respiratory chain linked to sulfide and acetate oxidation: NAD(P)H dehydrogenase, cytochromes of the a, b, c, and CO-binding types, and ubiquinone Q8 (8, 93). Acetate and sulfide oxidation appear to involve the same or similar respiratory chains, except that ubiquinone Q8 is used in acetate oxidation but not in sulfide oxidation (79). Acetate is also assimilated as a carbon source and is stored as poly-beta-hydroxybutyrate (21, 88). Although autotrophic growth has never been demonstrated for B. alba B18LD (66, 88, 90), the enzymes

ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase, which are normally associated with the Calvin cycle of autotrophic carbon dioxide fixation, have recently been discovered in this organism (66). No physiological roles in B. alba have been proposed for these enzymes.

B. alba B18LD can use dinitrogen gas, nitrate, nitrite, ammonium, urea, asparagine, aspartate, alanine, and thiourea as sole nitrogen sources (65, 99, 100). It cannot use methionine or glutamate as nitrogen sources (99). High glutamine synthetase and glutamate synthase activities are present when cells are grown with both low and high external concentrations of ammonium, and methionine sulfoximine, an inhibitor of glutamine synthetase, prevents growth at both ammonium concentrations, suggesting that ammonium assimilation in B. alba B18LD occurs primarily by the glutamine synthetase-glutamate synthase pathway (99). This is supported by the observation that glutamate dehydrogenase activity is lacking (99). In other bacteria, glutamine synthetase and glutamate synthase act in conjunction to assimilate ammonia at low ammonia concentrations, and glutamate dehydrogenase is primarily responsible for ammonia assimilation at high ammonia concentrations (54). Low alanine dehydrogenase activity is present in B.

alba B18LD growing at high ammonium concentrations and may be a minor pathway for ammonium assimilation (99). Nitrate is assimilated by B. alba B18LD and is not used as an alternate electron acceptor to oxygen (100). Ammonia appears to be an intermediate in nitrate assimilation (100). The ability of B. alba B18LD to fix N_2 has previously been demonstrated by showing that the organism could grow in the absence of combined nitrogen, that it could reduce acetylene to ethylene during such growth, and that acetylene reduction is prevented in the presence of combined nitrogen (65).

In this study, characteristics of in vivo nitrogenase activity in B. alba B18LD were investigated. A methodology by which B. alba nitrogenase activity could be induced in cells pre-grown on NH_4Cl was devised. The procedure entails washing the cells free of exogenous ammonium, resuspending the cells in nitrogen-deficient broth at a higher cell concentration than the original culture, dispensing the cells to sealed reaction vessels, and lowering the amount of oxygen present in the headspaces of such vessels to a level which would maximally support acetylene reduction by nitrogenase. Using this induction methodology, acetylene reduction by B. alba was characterized according to: (1) pH and temperature tolerance and (2) repressive and

inhibitory effects of nitrogenous compounds. Immediate inhibition of B. alba nitrogenase activity by ammonium was observed and this was investigated in greater detail. Short-term ammonium-induced inhibition of in vivo nitrogenase activity has previously been studied with other nitrogen-fixing bacteria, including members of the genera Azotobacter (41, 43), Azospirillum (27), and Rhodospirillum (37, 38, 52, 70). Recently, ammonium inhibition of nitrogen fixation in the rhizosphere of a salt marsh grass, Spartina alterniflora, was demonstrated (104). Ammonium inhibition of B. alba nitrogenase activity is relevant to this, since Beggiatoa inhabits the root zone of Spartina alterniflora (90).

MATERIALS AND METHODS

Induction of nitrogenase activity.

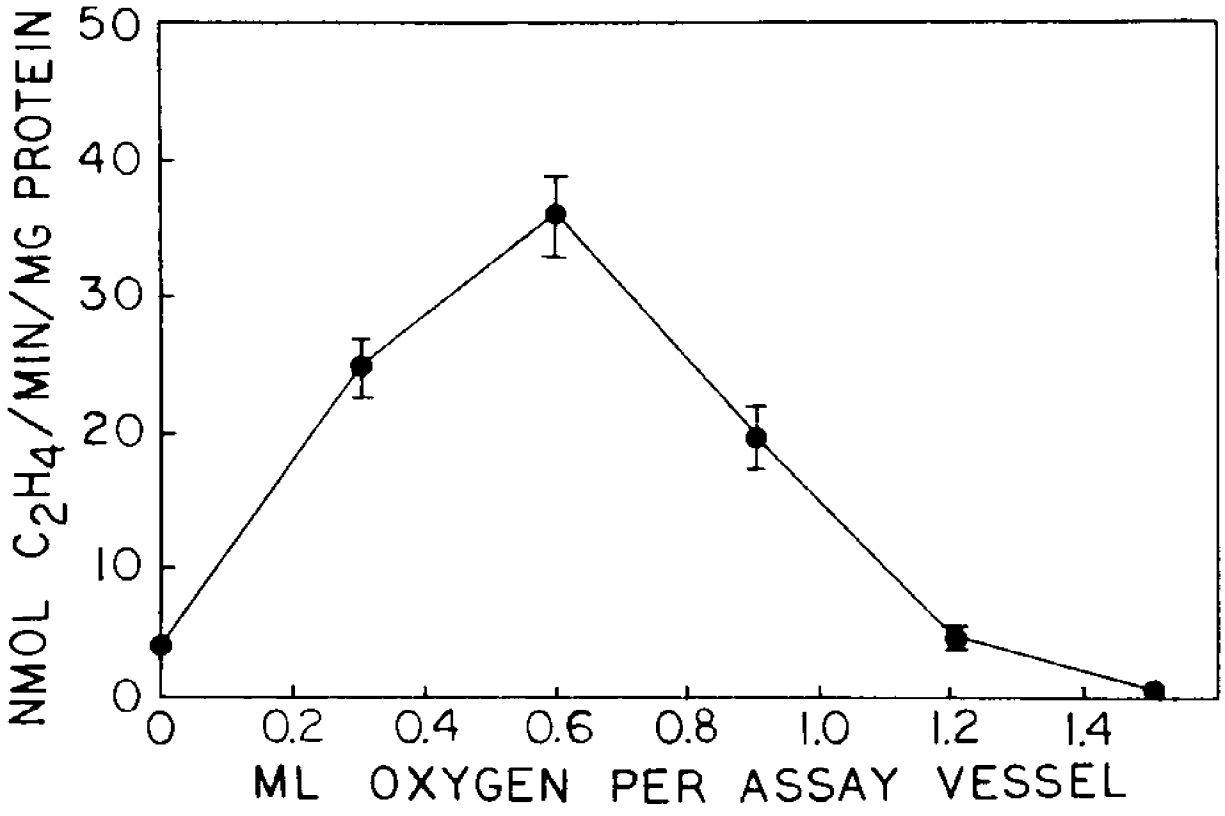
Beggiatoa alba B18LD cells to be used for nitrogenase induction were initially grown non-diazotrophically in modified AC medium at 29°C, shaking at 150 rpm, as previously described (88). This medium is similar to the modified MP05 medium described in Part I, except that sodium sulfide is omitted. It contains acetate as the sole energy and carbon source and NH_4Cl as the sole nitrogen source. The nitrogen-deficient medium used in nitrogenase assays, hereafter referred to as NDAC, was similar to modified AC medium, except that ammonium chloride was omitted, and the concentrations of $MgSO_4$ and Na_2MoO_4 were increased to 0.8 mM and 1 μM, respectively (83).

Logarithmic phase cells, grown in AC medium as described above, were washed and resuspended in NDAC medium to a concentration at which the optical density at 660 nm was equal to 0.570 +/- 0.020. 5 ml portions of cell suspension were put into 60 ml serum bottles which were then closed with rubber seals. The headspace gas in the bottles was replaced with helium by continuously flushing the bottles with helium for 30 minutes. 6 ml of headspace gas was removed from each bottle and

immediately replaced with 6 ml of acetylene. Quantities of pure oxygen which ranged from 0 to 1.5 ml were added to the bottles. The vessels were shaken at 150 rpm and 29°C. Ethylene was quantified by gas chromatography 2 hours later. After ethylene measurement, 1 ml aliquots of cell suspension were centrifuged, resuspended in 1 N NaOH, and lysed by heating at 90°C for 10 minutes. The lysate was neutralized with 1 N HCl, and then analyzed for protein as previously described in Part I. Figure 1 illustrates that 600 µl of oxygen added initially resulted in the highest rate of acetylene reduction. Oxygen levels above and below this resulted in lower acetylene reduction rates (Figure 1). All future experiments were performed using this quantity of oxygen, and this procedure is referred to in the remainder of this work as the "induction method," "induction procedure," or as the "induction of nitrogenase activity." The induction method provided stable and linear acetylene reduction by cells and was used for all of the experiments described below which involved acetylene reduction.

To test if the induction method reflects the ability of the organism to fix N_2, the following experiment was performed. 30 ml of cell suspension, prepared as described above, was added to each of two 120 ml serum

Figure 1. Induction of nitrogenase activity at different oxygen concentrations. X-axis values denote volumes of oxygen added per 60 ml serum bottle at the onset of the induction method. Each data point is an average of duplicate or triplicate experiments. Error bars represent the standard deviations of the mean values.


NMOL C_2H_4/MIN/MG PROTEIN
50
40
30
20
10
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
ML OXYGEN PER ASSAY VESSEL

bottles, which were then sealed. One bottle was degassed with nitrogen, and one with helium. Each bottle received 5.4 ml oxygen. The bottles were then shaken at 150 rpm and 29°C, and three 0.5 ml cell suspension samples were removed from each bottle with a hypodermic needle and syringe every 2 hours for 8 hours. The samples were analyzed for protein content as described above.

Effects of chloramphenicol and nitrogenous compounds on nitrogenase induction. The repressive effects of chloramphenicol and various nitrogenous compounds were tested by adding them to different 5 ml aliquots of cell suspension just before the serum bottles were degassed in the induction procedure described above. Ethylene was quantified for each reaction vessel three hours after the addition of acetylene.

Effects of temperature and pH. Nitrogenase assays were performed as above, except that 2.5 ml of cell suspension and 250 µl of oxygen were added per 60 ml serum bottle. For temperature experiments, cells were induced at 29°C. 2 hours after the induction procedure was begun, the assay vessels were placed in stationary positions in incubators at temperatures ranging from 15 to 45°C. After temperature stabilization (30 minutes to 1 hour), acetylene reduction rates were measured. For pH experiments, pH values were held constant (+/- 0.2 pH

units) during the entire experiment using these biological buffers: 2(N-morpholino)-ethanesulfonic acid (MES), effective pH buffering range 5.5 to 6.7; 1,3-bis-tris(hydroxymethyl)methylaminopropane (bis Tris propane), effective pH buffering range 6.3 to 9.5; and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), effective pH range 6.8 to 8.2, all at final concentrations of 25 mM. Cellular protein was quantified for each assay as described above.

Immediate effects of nitrogenous compounds and other chemicals on nitrogenase activity. Nitrogenase activity was induced in replicate 5 ml cell suspension samples. Headspace samples were analyzed periodically for ethylene, and, at some point in the testing period, various compounds that were dissolved in water or acetone, such as ammonium chloride, urea, amino acids, KCl, CsCl, TlCl (thallium chloride), PMS (phenazine methosulfate), DCCD (dicyclohexylcarbodiimide), or sodium arsenate, were injected in 100 or 200 µl volumes into the assay vessels. This volume of water or acetone injected by itself had no effect on the nitrogenase activity. In some cases glutamine or MSX (methionine sulfoximine) was added prior to NH_4Cl, TlCl, or PMS.

Growth experiments with glutamine and other chemicals. Duplicate or triplicate AC broth cultures of

B. alba were shaken at 150 rpm, 29°C. Glutamine, sodium arsenate, or DCCD was included in one of these cultures from the outset of the incubation. Growth was monitored by measuring optical density at 660 nm over a 72 hour period.

Ammonium uptake by cells. Ammonium uptake was analyzed by monitoring the disappearance, from the culture medium, of added ammonium. Cell suspensions were prepared as in the induction procedure. One experiment was designed to monitor acetylene reduction and ammonium uptake simultaneously. 55 ml of cell suspension was added to a 500 ml reaction vessel, and the headspace gas was adjusted with helium, oxygen, and acetylene as described in the induction procedure. After 2 to 3 hours of shaking at 150 rpm and 29°C, NH_4Cl was injected into the cell suspensions and 3.5 ml cell samples were removed periodically with a hypodermic needle and syringe and were filtered immediately with disposable syringe filters (cellulose acetate, 0.22 um pore size). The filtrates were deposited in glass test tubes. Acetylene reduction was monitored throughout the experiment. Filtrates were analyzed for ammonium content by the indophenol blue reaction (26), in the following manner. 2.5 ml of a filtrate sample was transferred to a 50 ml test tube. 25 µl of 1.5 mM $MnSO_4 \cdot H_2O$ was added, and

mixed in by vortexing. 125 µl of a 20% (v/v) aqueous solution of household bleach, pH 7, was added while vortexing the tube. Immediately after this, 150 µl of a solution containing 10% (w/v) phenol in 0.625 M NaOH was added dropwise while still vortexing. The tube was set at room temperature for 30 minutes to 1 hour, and then the absorbance at 630 nm was measured. Aqueous ammonium solutions of various concentrations were analysed similarly to prepare a standard curve from which the ammonium concentrations of the filtrate samples were determined. Other uptake experiments were designed to test the effects of MSX, glutamine, and TlCl on ammonium uptake and were performed similarly, except that 30 ml of cell suspension was used per 120 ml serum bottle and acetylene was not included in the headspace gas. MSX, glutamine, or TlCl was added 15 to 30 minutes prior to NH_4Cl. It was found that the following components caused negligible interference with the specificity of the indophenol blue assay for ammonium: MSX, glutamine, AC medium, and TlCl.

Degree of ammonium- and thallium-induced inhibition at different constant pH values. Cell suspensions were induced as described above except that 25 mM bis Tris propane buffer was included to stabilize the external pH at different values ranging from pH 7 to

8. The effects of ammonium chloride and TlCl on the nitrogenase activities of these suspensions were tested as described above.

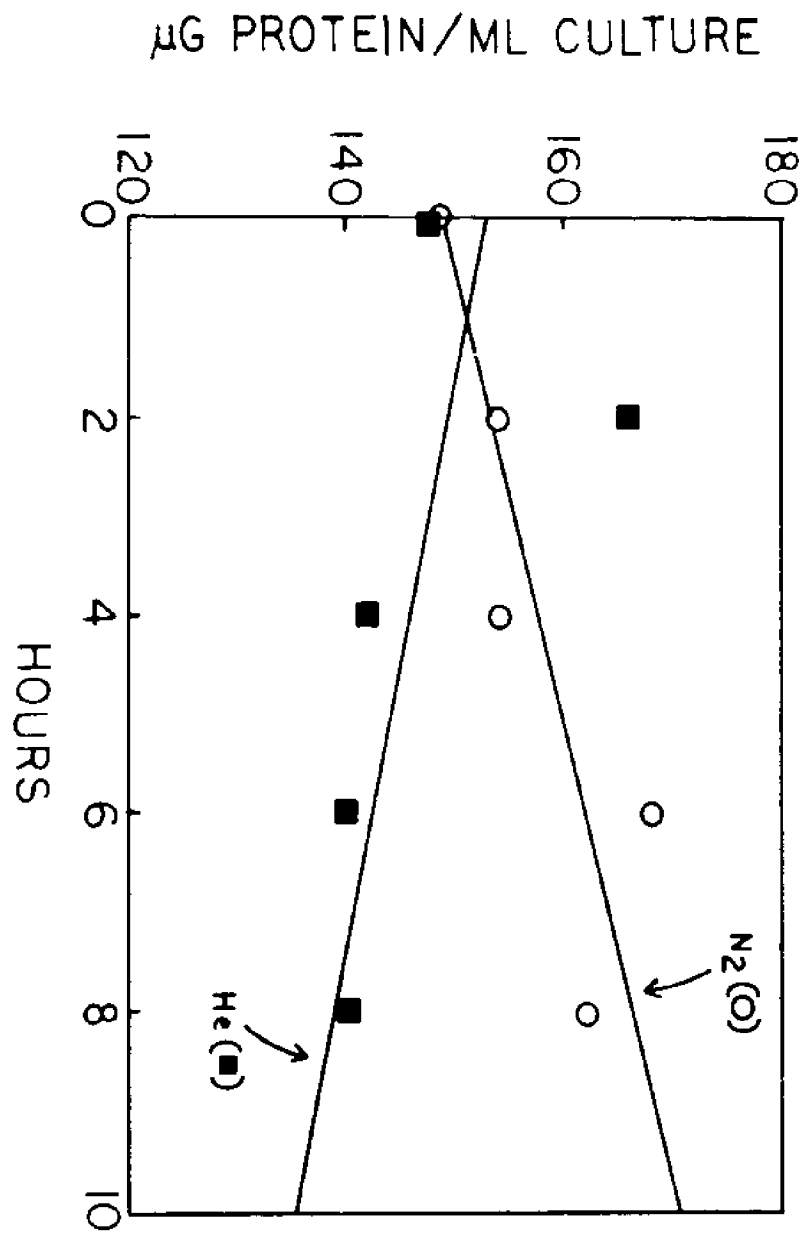
Chemicals. MSX, DCCD, PMS, amino acids, and buffers were obtained from Sigma Chemicals. TlCl was obtained from Aldrich Chemicals. Helium and nitrogen gases were supplied by Big Three Industries, Inc. Acetylene was produced by the addition of calcium carbide to distilled water, as described in detail in Part I.

RESULTS AND DISCUSSION

Validity of the induction method as a nitrogen fixation assay. As Figure 2 shows, using the induction method, cellular protein increased in cultures exposed to N_2 as compared to a helium control. This implies that the cells were fixing the N_2, and using it as a nitrogen source and, that the use of the induction procedure and the acetylene reduction assay described in Materials and Methods is valid as a means of analyzing diazotrophy in Beggiatoa alba B18LD. Our observation that B. alba nitrogenase activity was highest in the presence of a small amount of oxygen (see Materials and Methods, Figure 1) is in agreement with the microaerophilic diazotrophic growth observed for Beggiatoa alba B18LD cultivated in sulfide-oxygen gradients (65). Although B. alba nitrogenase is apparently oxygen-sensitive, the organism requires a small amount of oxygen for maximal nitrogen fixation (Figure 1). In this respect, B. alba is similar to the nitrogen-fixing Azospirillum (27). The induction of nitrogenase in B. alba and in Rhizobium meliloti are similar in that both occur primarily under microaerobic conditions (10, 11, 23).

Effect of chloramphenicol and nitrogenous compounds on nitrogenase induction. Induction of nitrogenase was

Figure 2. Growth of nitrogenase-induced cultures on dinitrogen gas. Duplicate cultures were degassed with either N_2 (O) or helium (■). Small amounts of oxygen were added to each in order to mimic the microaerobic conditions of the induction method, and growth was monitored over an 8 hour period. Best-fit lines were derived from a linear regression analysis of the data points performed with a Casio fx-7000G graphics calculator.


µG PROTEIN/ML CULTURE
120
140
160
180
0
2
4
6
8
10
HOURS
He (■)
N2 (○)

prevented in cells that were treated before induction with chloramphenicol, a protein synthesis inhibitor (Table 1). The ability of chloramphenicol to prevent nitrogenase induction indicates that B. alba nitrogenase is not synthesized during the classically non-diazotrophic conditions (high oxygen and ammonium levels) under which the cells were grown prior to treatment by the induction method. Nitrate and nitrite also prevented induction of nitrogenase (Table 1). None of these compounds affected nitrogenase activity when added after cells had begun reducing acetylene (Figure 3, Table 2). This indicates that nitrate and nitrite probably cause repression of nitrogen fixation genes in B. alba. This has been observed with other bacteria and may occur after the conversion of nitrate to ammonia or by a mechanism which is separate from ammonia-induced repression (72). Glutamine stimulated nitrogenase induction (Table 1). Enhancement of induction and activity is probably due to the ability of glutamine to function as a nitrogen source, since, in our experiments, the cells were nitrogen-starved. The inability of glutamine to repress nitrogenase suggests that ammonium assimilation products do not negatively regulate B. alba nif genes, as they do in some other bacteria (23). Rhizobium meliloti is similar to B. alba in this respect

Table 1. Inhibition of nitrogenase induction.

Compound added[a]	nmol C_2H_4/assay vessel[b]
none	267 +/- 37
CAP[c] (20 µg/ml)	0
$NaNO_3$ (2mM)	0
$NaNO_2$ (2mM)	0
glutamine (10 mM)	461 +/- 25
glutamine (20 mM)	498 +/- 32

[a] Compounds were added to non-diazotrophic cell suspensions at the start of the nitrogenase induction procedure described in materials and methods section.

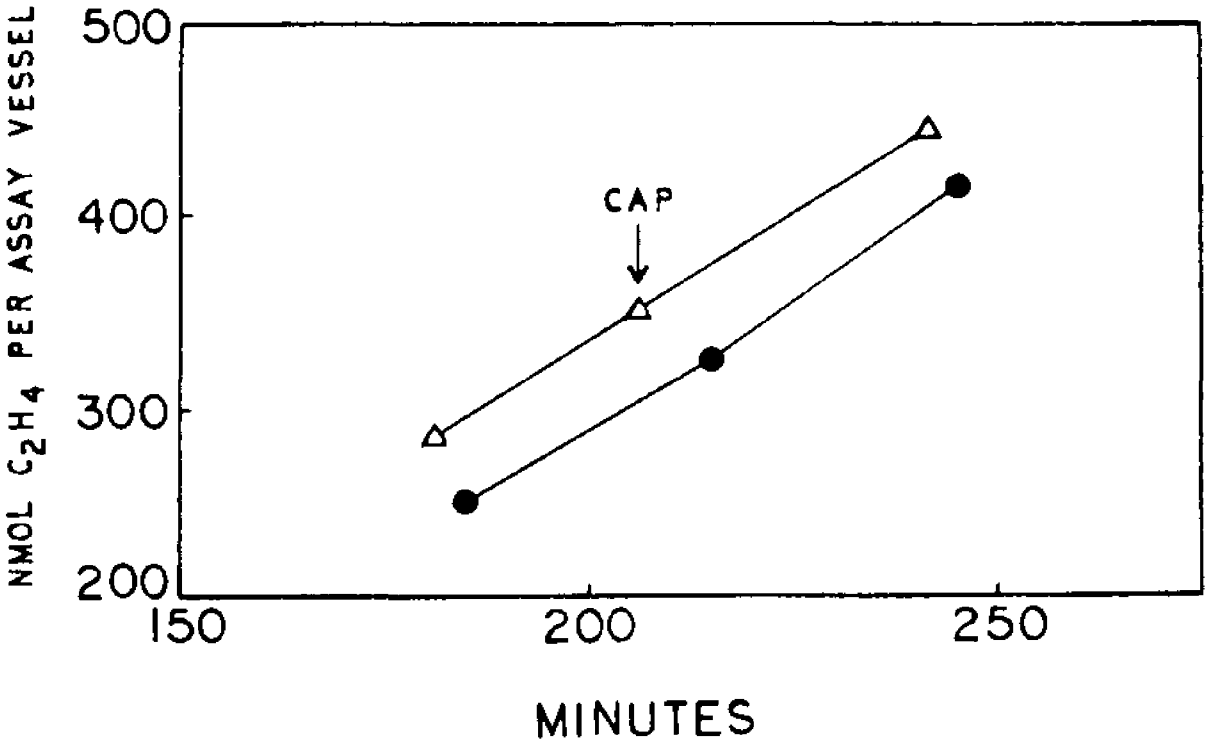
[b] Ethylene was quantified 3 hours after acetylene injection. Means and standard deviations were derived from duplicate assays.

[c] CAP, chloramphenicol.

Table 2. Immediate inhibition of nitrogenase activity by nitrogenous compounds.

Compounds which:	
Inhibit	Do not inhibit
NH_4Cl	glutamate
$(NH_4)_2SO_4$	glutamine
hydrazine sulfate	aspartate
hydroxylamine·HCl	asparagine
urea	alanine
	proline
	methionine
	arginine
	lysine
	leucine
	histidine
	glycylglycine
	KNO_3
	$NaNO_2$

Figure 3. Immediate effect of chloramphenicol (CAP) on in vivo acetylene reduction. CAP was added at the time indicated by the arrow to one of two replicate acetylene-reducing cell suspensions. The symbol Δ denotes the culture which received CAP at a final concentration of 20 µg/ml, and the symbol ● denotes the culture which did not receive CAP.


NMOL C_2H_4 PER ASSAY VESSEL
500
400
300
200
150
200
250
CAP
MINUTES

because ammonium metabolism does not appear to be intimately related to the regulation of R. meliloti nif genes (10, 11, 23).

Temperature and pH tolerance. Nitrogenase activity was highest at 29°C, extremely low at 15 and 37°C, and was undetectable at 45°C (Figure 4). The observed temperature tolerance is probably indicative of the optimum growth temperature for the organism, which grows at 0 to 38°C but not at 42°C (58), and of the temperature sensitivity of biological nitrogen fixation in general (72).

Using bis Tris propane, MES, and HEPES buffers, we observed that the optimum pH range for nitrogenase induction and activity was between 6.5 and 8.0 (Figures 5 and 6). Activity was undetectable at pH 6.0 and 9.2 (Figure 5). A broad pH optimum for nitrogenase activity (6.9 - 8.0) has also been reported for Rhodopseudomonas sulfidophila (39). The lack of activity at the pH extremes of 6.0 and 9.2 is probably due to the inability of B. alba to grow, and thus produce nitrogenase, at these pH values.

Immediate effects of nitrogenous compounds. Ammonium chloride, ammonium sulfate, and urea caused immediate inhibition of nitrogenase activity when added to acetylene-reducing cell suspensions (Table 2).

Figure 4. Nitrogenase activity at different temperatures. Replicate acetylene-reducing cell suspensions were incubated at various temperatures and in vivo nitrogenase rates were determined.

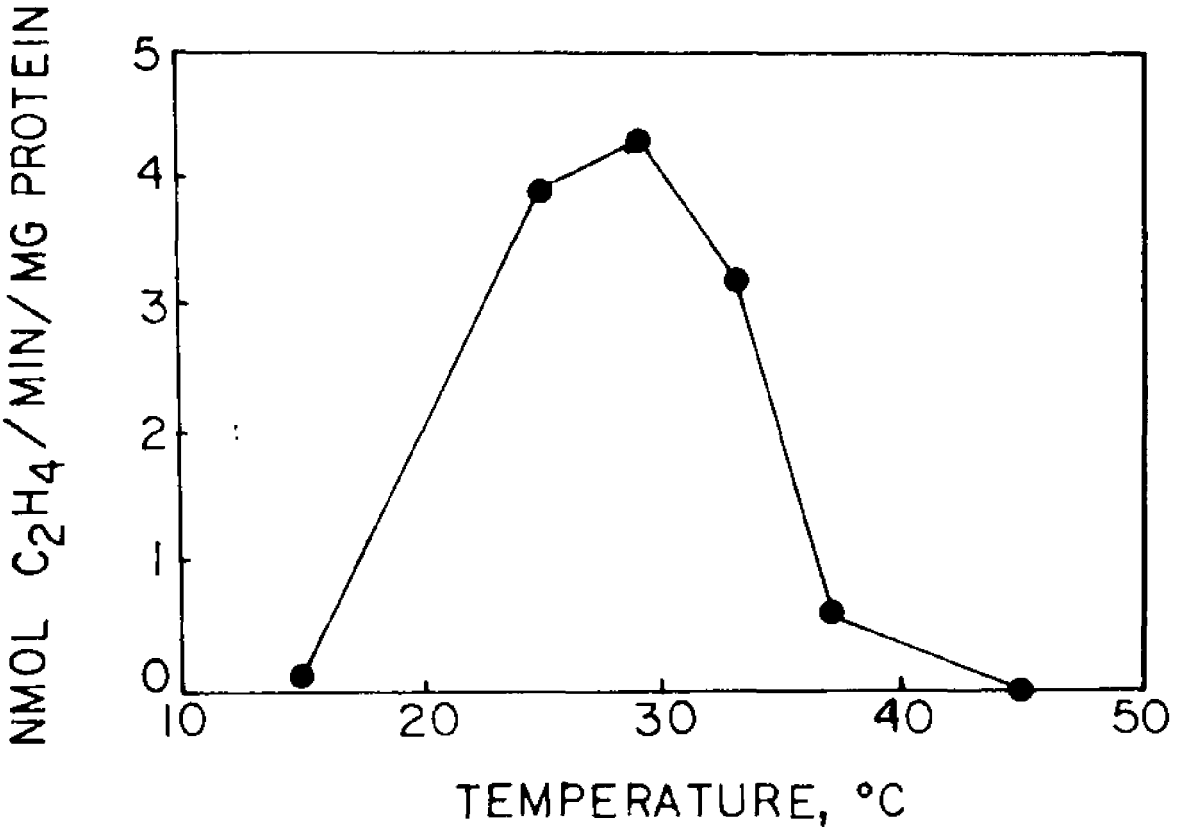

NMOL C_2H_4 / MIN / MG PROTEIN
0
1
2
3
4
5
10
20
30
40
50
TEMPERATURE, °C

Figure 5. Induction of in vivo nitrogenase activity at different pH values. External pH values were held constant with MES (Δ) and bis Tris propane (●) buffers. For enzyme rates derived using bis Tris propane, each data point is an average of two rate measurements. Error bars represent the standard deviations of the mean values. For enzyme rates derived using MES, each data point represents a single rate calculation.

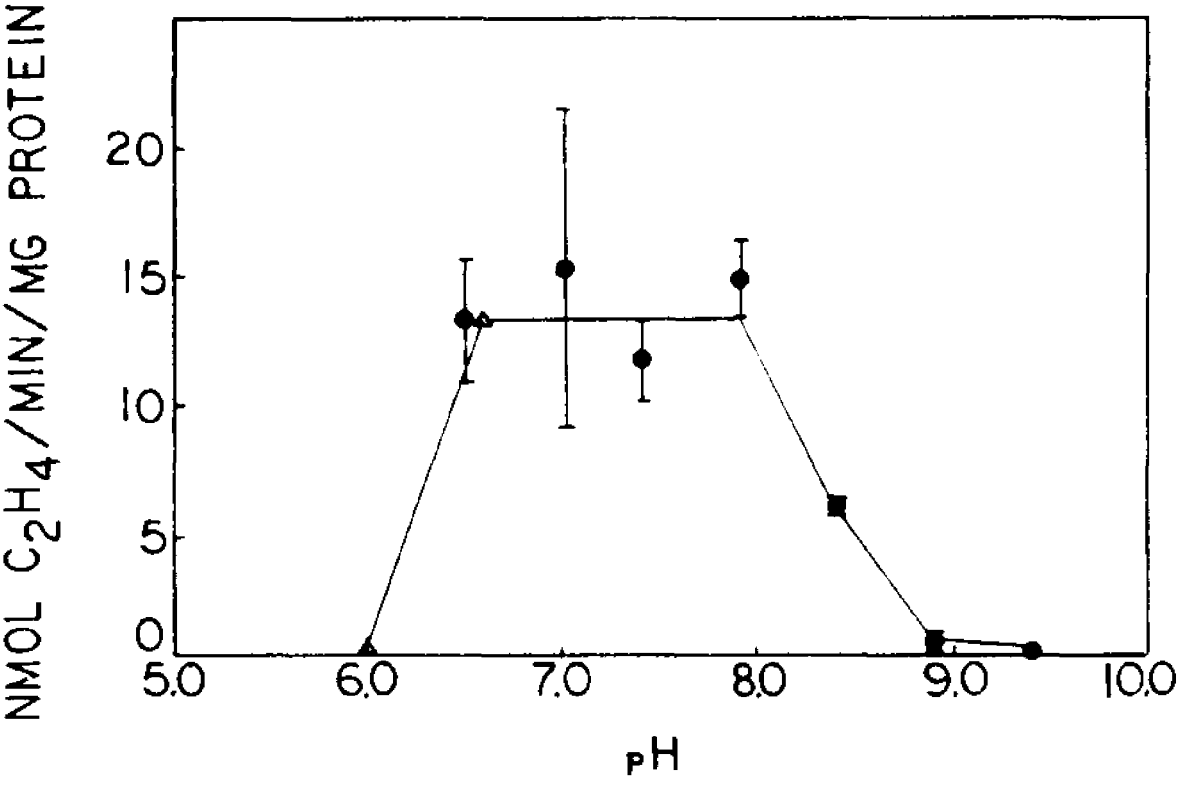
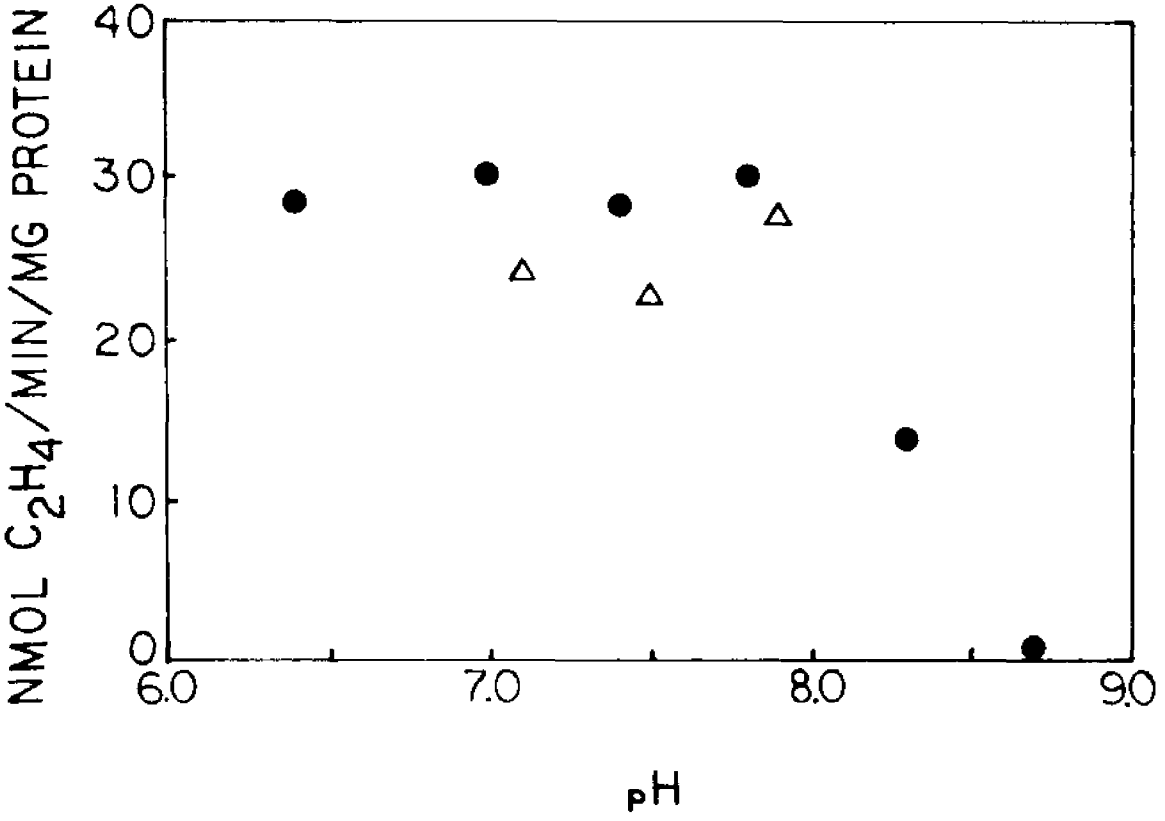

NMOL C_2H_4/MIN/MG PROTEIN
20
15
10
5
0
5.0
6.0
7.0
8.0
9.0
10.0
pH

Figure 6. Induction of in vivo nitrogenase activity at different pH values. External pH values were held constant with HEPES (Δ) and bis Tris propane (●) buffers. Each data point represents a single rate calculation.


NMOL C_2H_4/MIN/MG PROTEIN
40
30
20
10
0
6.0
7.0
8.0
9.0
pH

$NaNO_2$, KNO_3, and several amino acids including glutamine, glutamate, asparagine, and aspartate did not cause an immmediate inhibition of enzyme activity (Table 2). Since it was curious that ammonium caused an immediate inhibition but glutamine, the primary assimilation product of ammonium (99), did not, the ability of B. alba to use glutamine as a nitrogen source was tested. Growth studies indicated that it was able to use glutamine as a sole nitrogen source (Figure 7). The inability of glutamine to inhibit nitrogenase activity occurred whether the cells used for nitrogenase assays were originally grown on NH_4Cl or glutamine, which ruled out the possibility that the ability to utilize glutamine had to be induced in order for glutamine to have an effect on nitrogenase activity. B. alba may be incapable of transporting some of the other amino acids which were not inhibitory, although it is known that aspartate, asparagine, and alanine can serve as sole nitrogen sources for the growth of this organism (99).

The inhibitory period created by 0.1 mM NH_4Cl lasted approximately twice as long as that created by 0.05 mM NH_4Cl (compare Figures 8 and 9). Inhibition was incomplete at both concentrations. The degree of inhibition appeared to be maximal at 1.0 mM and at this concentration, inhibition was also incomplete (Figure 8).

Figure 7. Growth on different nitrogen sources. B. alba B18LD cells were cultivated in AC medium in shaker flasks with either 3.7 mM ammonium chloride, ● , or 1 mM glutamine, □ , as a sole nitrogen source. The symbol ▲ denotes a control which had no nitrogen source.

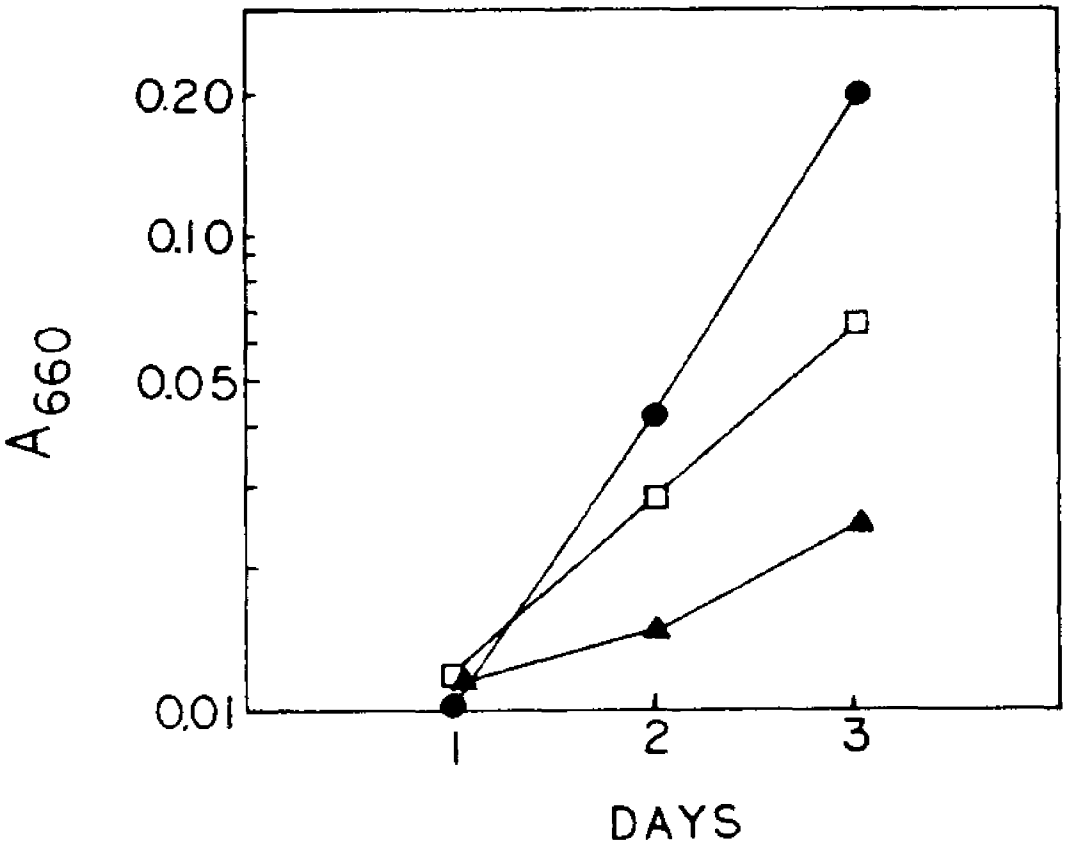

0.20
0.10
0.05
0.01
A660
1
2
3
DAYS

Figure 8. Ammonium chloride inhibition of in vivo nitrogenase activity. Acetylene-reducing cultures received the following additions: ● ,none; ▲ , NH_4Cl, final concentration 0.1 mM; □ ,NH_4Cl, final concentration 1 mM. The arrow indicates the time at which NH_4Cl was added.

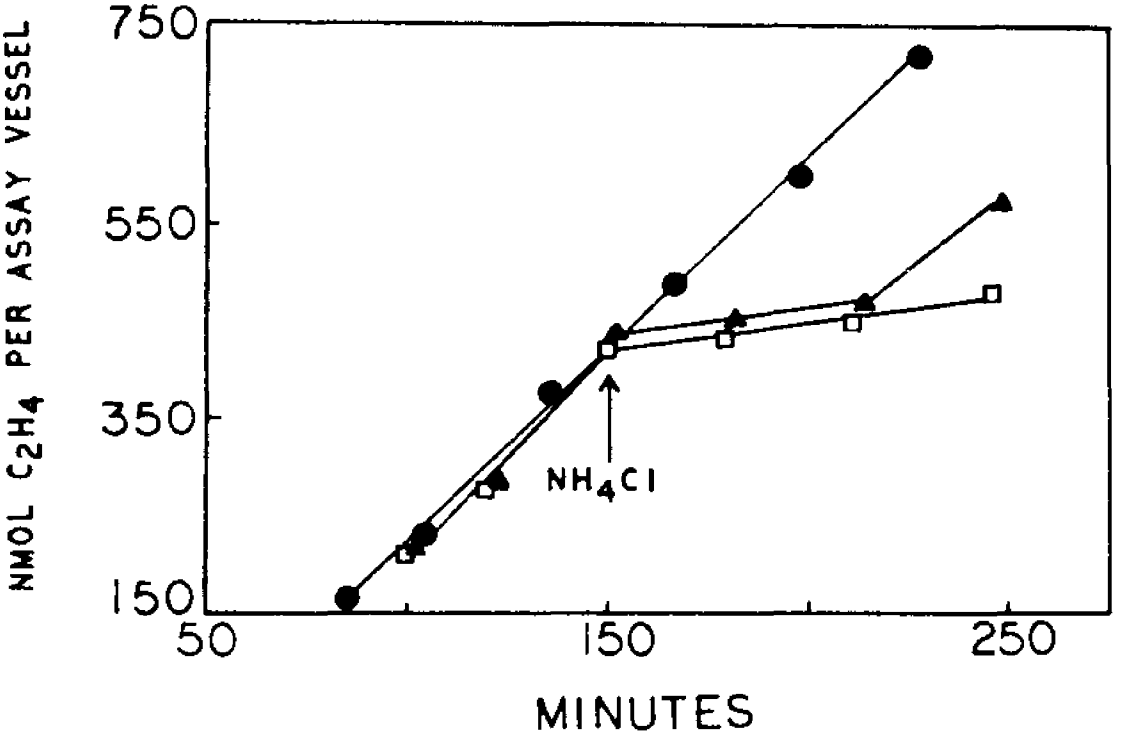
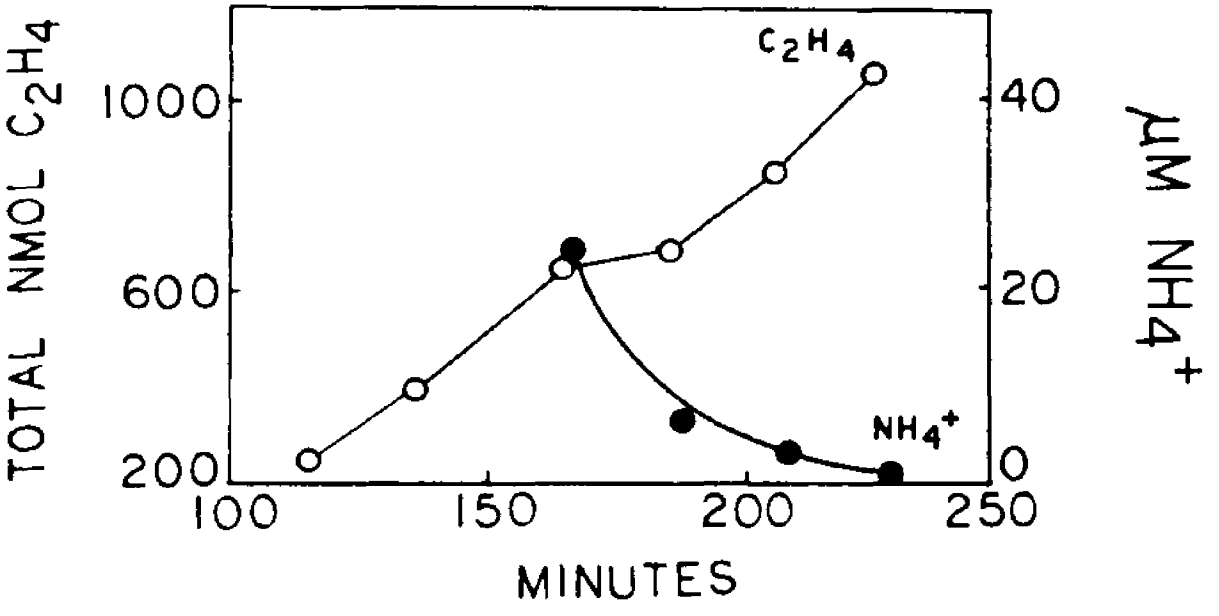

750
550
350
150
50
150
250
MINUTES
NMOL C_2H_4 PER ASSAY VESSEL
NH_4Cl

Figure 9. Ammonium uptake during inhibition of nitrogenase activity. NH_4Cl, final concentration 0.05 mM, was added at the time indicated by the arrow to an acetylene-reducing culture, and culture samples were periodically removed and analyzed for ammonium disappearance from the medium. Open circles, ○ , indicate ethylene production and closed circles, ● , indicate ammonium concentration in the medium.


TOTAL NMOL C_2H_4
1000
600
200
100
150
200
250
MINUTES
C_2H_4
NH_4^+
40
20
0
μM NH_4^+

In two separate experiments, inhibition was also incomplete at concentrations of ammonium chloride ranging from 10 μM to 4 mM (Table 3). It is apparent from the unchanging slope of the line representing inhibition by 1 mM ammonium chloride in Figure 8 that inhibition is immediate without a gradual transition. Ammonium disappearance from the medium occurred during the inhibitory period, and nitrogenase activity resumed at an extracellular ammonium concentration of approximately 6 μM (Figure 9).

Ammonium inhibition of in vivo nitrogenase activity in Rhodospirillum rubrum, Azospirillum brasilense, and Azosprillum lipoferum involves a modification of the iron protein (dinitrogenase reductase) of nitrogenase (27, 37, 38). In Rhodospirillum rubrum this modification is an ADP-ribosylation of the iron protein by an inactivating enzyme (52, 70). Inhibition in these organisms is a gradual process characteristic of enzymatic action (27, 37). Ammonium inhibition of nitrogenase activity in B. alba is immediate without a gradual transition, implying that it is not an enzymatic process for this organism. This characteristic is similar to the ammonium inhibition of in vivo nitrogenase activity in Azospirillum amazonense, in which inhibition is immediate and does not result in iron protein modification (27). Another

Table 3. Percentage of inhibition of nitrogenase activity by different concentrations of ammonium chloride.[a]

NH_4Cl concentration	% Inhibition
Experiment #1[b]	
0.01 mM	0
0.1 mM	55
0.5 mM	66
1 mM	77
2 mM	71
Experiment #2	
1 mM	60
4 mM	61

[a] NH_4Cl was added to cell suspensions that were reducing acetylene, as described in material and methods section.

[b] Two experiments were done under similar conditions on different dates.

similarity in this phenomenon that occurs among B. alba, A. amazonense, and A. vinelandii, is that, at least in some reported cases, inhibition is incomplete (19, 27).

The inability of glutamine, asparagine, and other amino acids to inhibit nitrogenase activity by B. alba indicates that assimilation products are not involved in the ammonium-induced inhibition, since the glutamine synthetase-glutamate synthase pathway is the primary route of ammonium assimilation in this organism. This contrasts with the nitrogenase inhibition by glutamine observed in A. brasilense and A. lipoferum (27) and by glutamine and asparagine in R. rubrum, which also results in ADP-ribosylation of the Fe protein (38).

The addition of MSX, a glutamine synthetase inhibitor (101), at a final concentration of 50 μM, prior to the addition of ammonium chloride, did not prevent inhibition of nitrogenase activity (Figure 10). MSX at a final concentration of 1 mM prevented ammonium-induced inhibition of nitrogenase activity, but also inhibited nitrogenase activity partially by itself (Figure 11). 50 μM MSX was sufficient to block ammonium uptake (Figure 12). 1 mM MSX blocked ammonium uptake to a similar extent (Figure 13). The ability of glutamine to prevent ammonium inhibition when added before ammonium was also tested, since it inhibits ammonium uptake in other

Figure 10. Effect of 50 µM MSX on ammonium inhibition of nitrogenase activity. Acetylene-reducing cultures received the following additions: ○, none; △, NH_4Cl; ●, MSX, then NH_4Cl. An arrow indicates the time at which MSX, final concentration 50 µM, was added. Another arrow indicates the time at which ammonium chloride, final concentration 0.05 mM, was added.

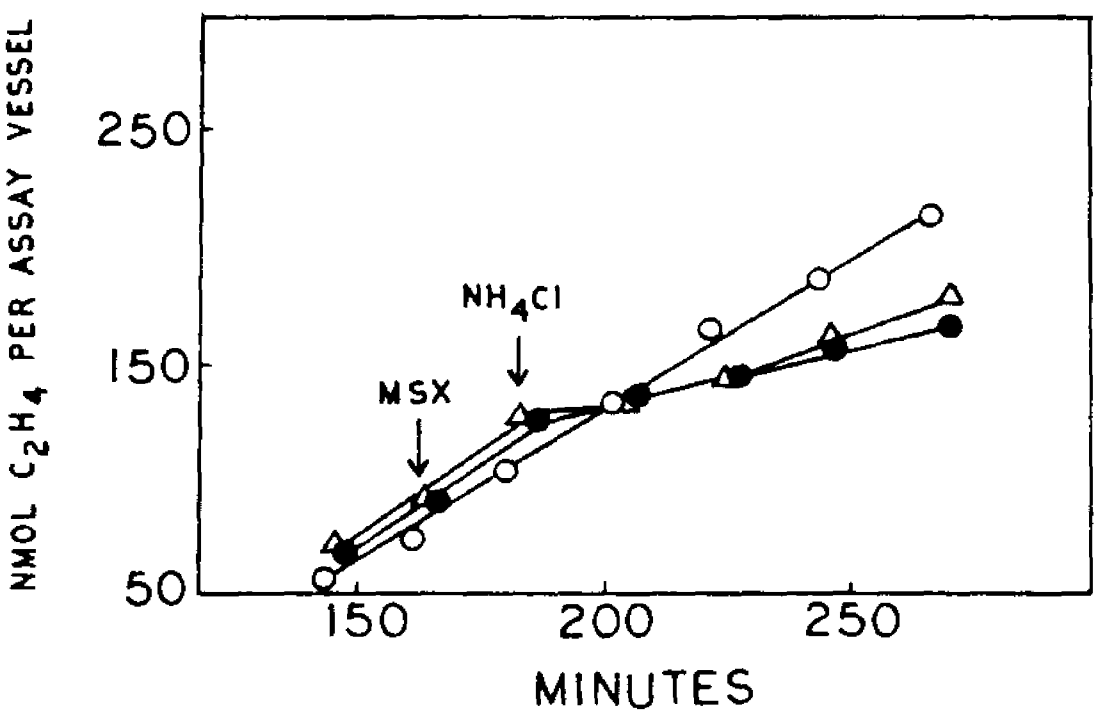

NMOL C_2H_4 PER ASSAY VESSEL
250
150
50
150
200
250
MINUTES
MSX
NH_4Cl

Figure 11. Effect of 1 mM MSX on ammonium inhibition of nitrogenase activity. Acetylene-reducing cultures received the following additions: ○, none; ■, NH_4Cl; △, MSX, then NH_4Cl. An arrow indicates the time at which MSX, final concentration 1 mM, was added. Another arrow indicates the time at which ammonium chloride, final concentration 0.1 mM, was added.

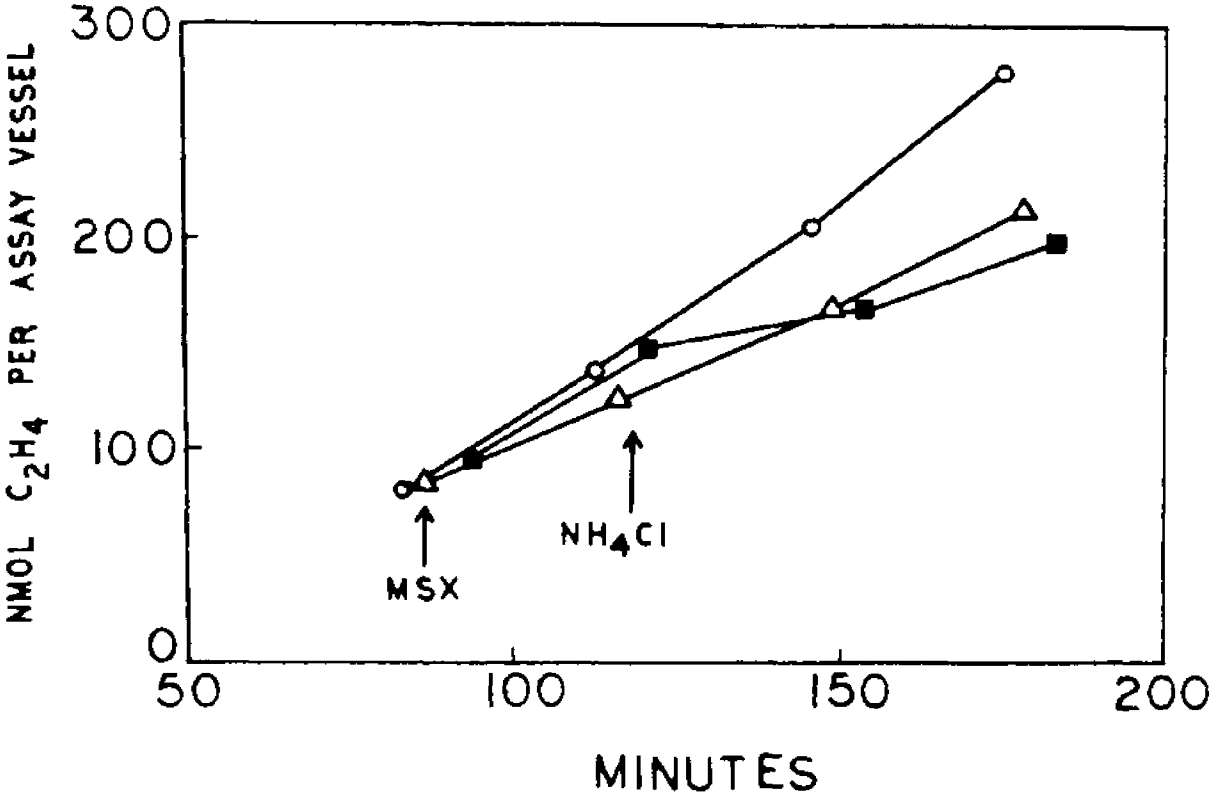

300
200
100
0
NMOL C_2H_4 PER ASSAY VESSEL
50
100
150
200
MINUTES
MSX
NH_4Cl

Figure 12. Inhibition of ammonium uptake by 50 uM MSX. The disappearance of ammonium from the medium of diazotrophic cultures, to which ammonium chloride, final concentration 0.05 mM, was added at time zero, was monitored. MSX, final concentration 50 μM, was added (△) or not added (●) 15 minutes prior to the addition of ammonium chloride.

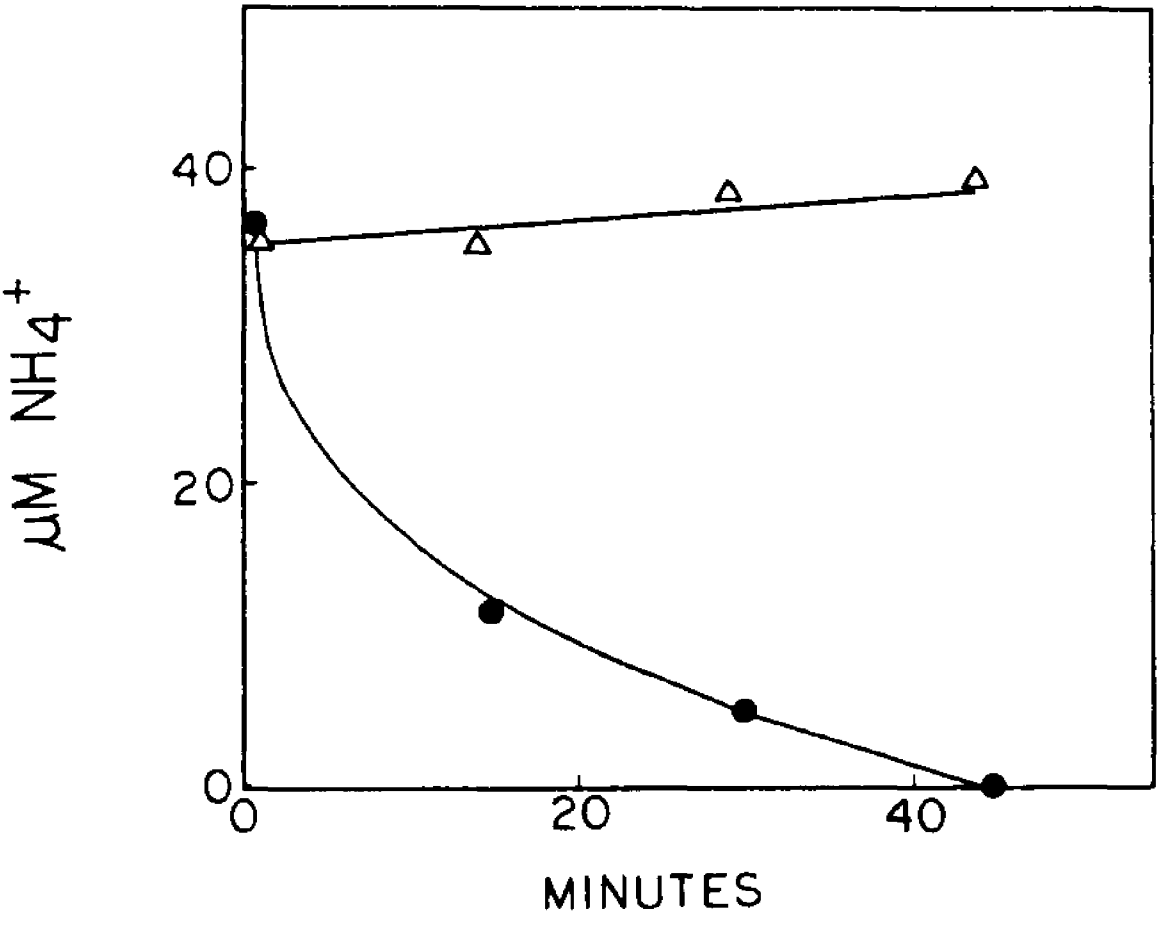
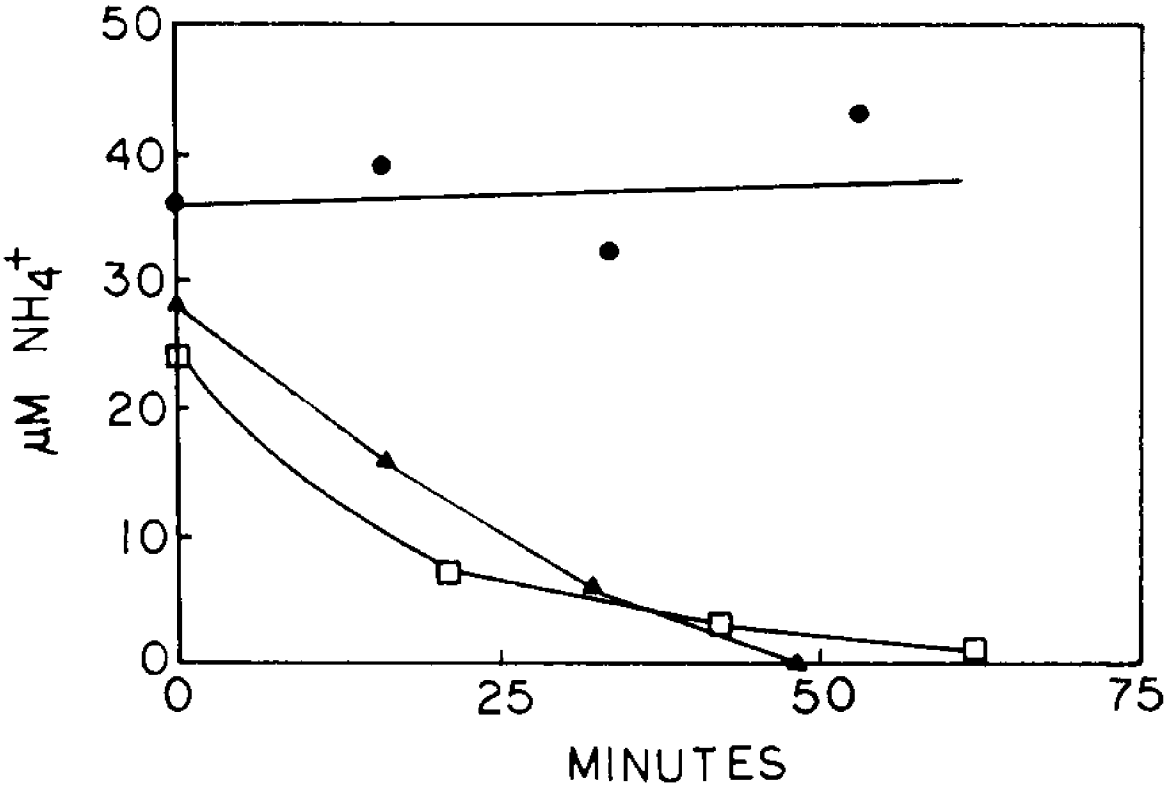

40
20
0
0
20
40
μM NH4+
MINUTES

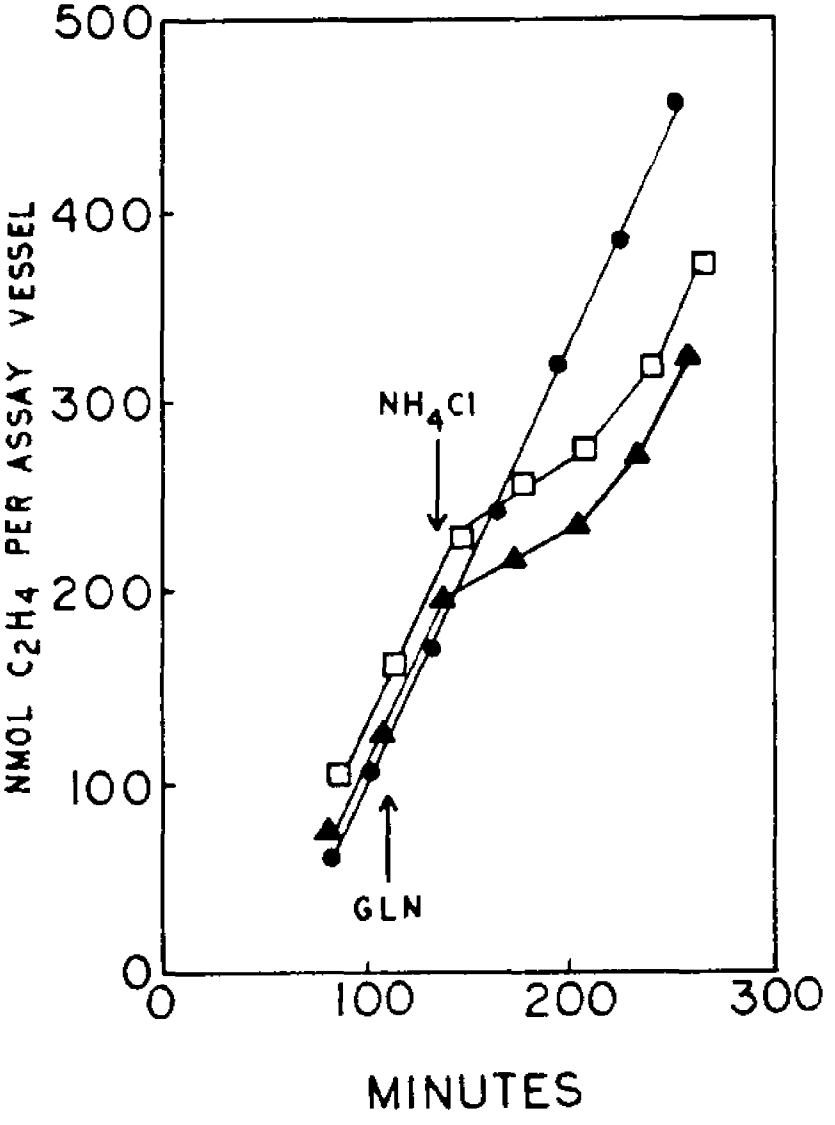
Figure 13. Effect of 1 mM MSX and glutamine on ammonium uptake. The disappearance of ammonium from the medium of replicate diazotrophic cultures, to which ammonium chloride, final concentration 0.05 mM, was added at time zero, was monitored. MSX, final concentration 1 mM, was added to one culture (●) 15 minutes prior to the addition of ammonium chloride. Glutamine, final concentration 1 mM, was added to another culture (▲) 15 minutes prior to ammonium chloride. Neither MSX nor glutamine was added to the culture denoted by the symbol □ .


50
40
30
20
10
0
μM NH4+
0
25
50
75
MINUTES

bacteria (30), but glutamine did not affect the inhibition (Figure 14). However, glutamine had little or no effect on ammonium uptake (Figure 13).

As mentioned above, 50 μM MSX does not prevent inhibition of B. alba nitrogenase by ammonium, and 1 mM MSX does prevent such inhibition, although both concentrations appear to be capable of blocking uptake of ammonium to the same extent. It is possible that at the lower concentration, MSX is merely inhibiting glutamine synthetase, and by doing so is inhibiting overall uptake of ammonium by the cell. This presumption is supported by the mechanism for bacterial ammonium transport that has recently been proposed which contends that once ammonium is transported into the cell it is converted into ammonia under the higher pH that is characteristic of the cell interior of a neutrophilic bacterium (40). The ammonia, which is an uncharged compound, is then free to passively diffuse back out of the cell if it is not used by glutamine synthetase. Once exterior to the cell, at a lower pH, the ammonia then becomes protonated to yield ammonium (40). Thus, although the ammonium ion is transported into the cell when low concentrations of MSX are present it continually diffuses back out, and so the overall uptake of ammonium by the cell is inhibited. At the higher concentration of MSX (1 mM), it is possible

Figure 14. Effect of glutamine on ammonium inhibition of nitrogenase activity. Acetylene-reducing cultures received the following additions: ● , none; □ , NH_4Cl; ▲ , glutamine, then NH_4Cl. An arrow indicates the time at which glutamine, final concentration 1 mM, was added. Another arrow indicates the time at which ammonium chloride, final concentration 0.1 mM, was added.


500
400
300
200
100
0
0
100
200
300
NMOL C_2H_4 PER ASSAY VESSEL
MINUTES
NH_4Cl
GLN

that ammonium transport, in addition to assimilation, is inhibited. If ammonium transport rather than assimilation were involved in the inhibition of nitrogenase activity, then this might explain why the inhibition is affected dissimilarly by different concentrations of MSX. Methylammonium is often used as a model compound for analyzing ammonium transport in bacteria (4, 40, 56) and it has been demonstrated that the degree to which MSX inhibits methylammonium transport in A. vinelandii depends on the concentration of MSX (30, 40). It was reported that 1 mM MSX inhibited methylammonium transport only 10%, and that 10 mM MSX increased the degree of inhibition to 60% (30, 40).

Since the data mentioned above appears to rule out the involvement of ammonium assimilation in the inhibition of nitrogenase activity, and suggests that ammonium transport might be involved, the effects of other ions on nitrogenase activity were tested. CsCl and KCl had no effect on nitrogenase activity (Figures 15 and 16). However, TlCl inhibited nitrogenase activity similarly to ammonium in that the inhibition was a rapid transition (Figure 17). Thallium inhibits ammonium transport in other bacteria (4, 28, 31). In E. coli thallium inhibition of ammonium permease is competitive (31). Therefore, it is possible that in B. alba, Tl^+ is

Figure 15. Immediate effect of cesium chloride on in vivo acetylene reduction. CsCl was added at the time indicated by the arrow to one of two replicate acetylene-reducing cell suspensions. The symbol △ denotes the culture which received CsCl at a final concentration of 10 mM, and the symbol ● denotes the culture which did not receive CsCl.

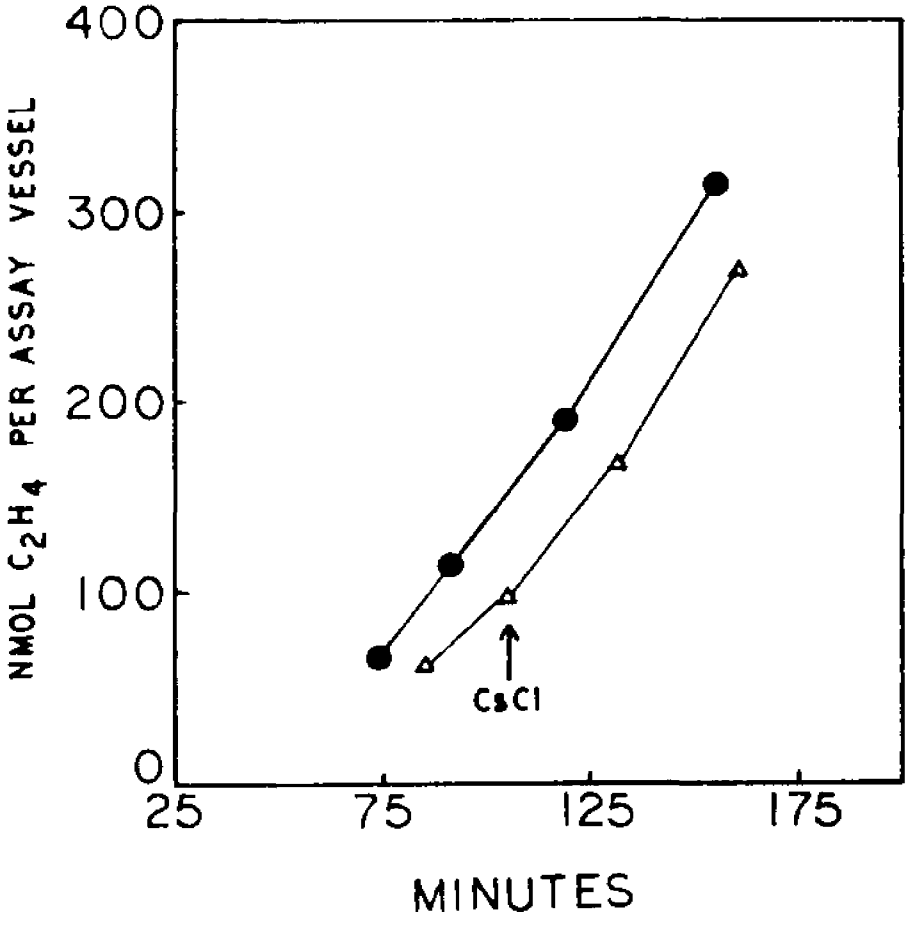

400
300
200
100
0
NMOL C_2H_4 PER ASSAY VESSEL
25
75
125
175
MINUTES
CsCl

Figure 16. Immediate effect of potassium chloride on in vivo acetylene reduction. KCl was added at the time indicated by the arrow to one of two replicate acetylene-reducing cell suspensions. The symbol □ denotes the culture which received KCl at a final concentration of 1 mM, and the symbol ● denotes the culture which did not receive KCl.

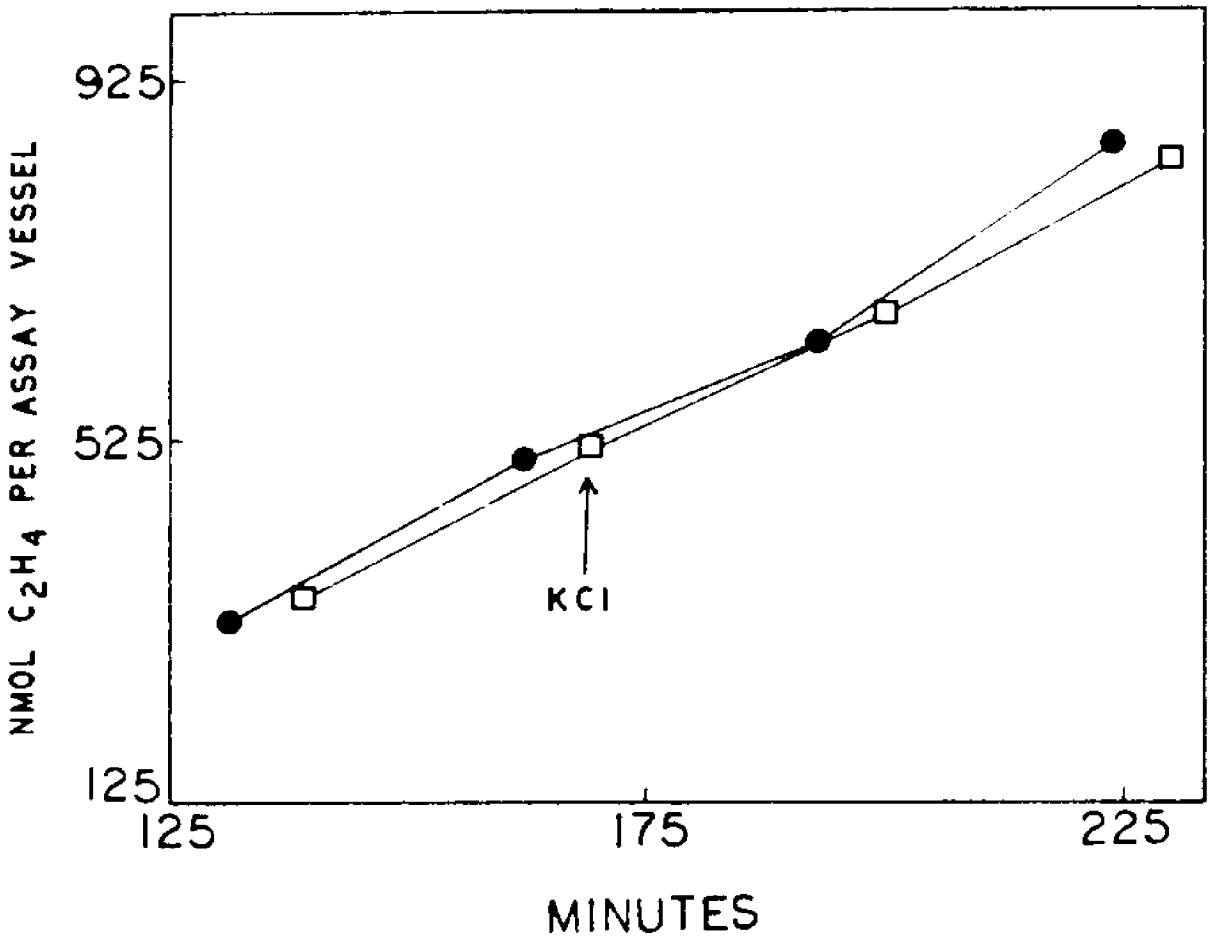
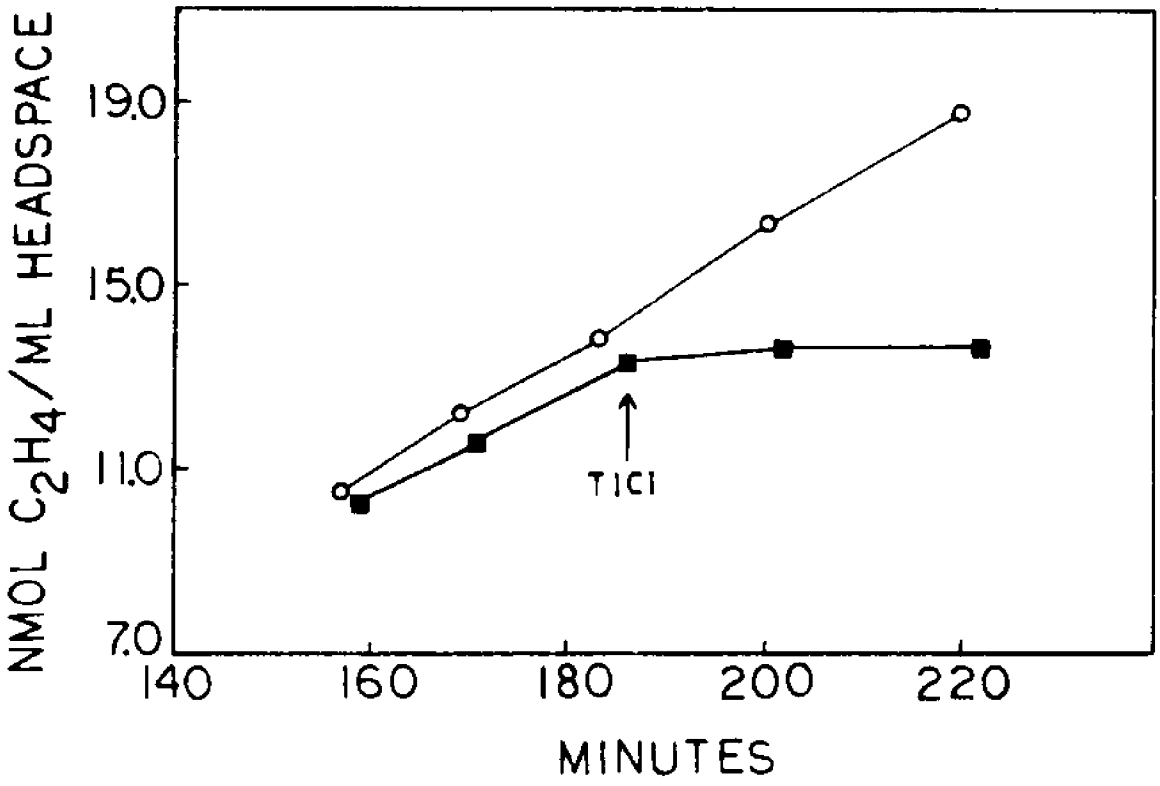

925
525
125
NMOL C_2H_4 PER ASSAY VESSEL
KCl
125
175
225
MINUTES

Figure 17. Immediate effect of thallium chloride on in vivo acetylene reduction. TlCl was added at the time indicated by the arrow to one of two replicate acetylene-reducing cell suspensions. The symbol ■ denotes the culture which received TlCl at a final concentration of 50 μM, and the symbol ○ denotes the culture which did not receive TlCl.


NMOL C_2H_4/ML HEADSPACE
19.0
15.0
11.0
7.0
140
160
180
200
220
MINUTES
TICI

transported into the cell by an ammonium permease, and thus has the same effect on nitrogenase activity that ammonium does. Different experiments were performed to determine if Tl^+ and NH_4^+ transport in B. alba are related. In one experiment, it was determined that 50 μM TlCl blocks ammonium uptake almost completely (Figure 18). This supports the idea that ammonium and thallium share the same permease for transport, and demonstrates that even if they do not, they at least share a common driving force for transport. Table 4 shows that 1 mM MSX decreased the degree of TlCl-induced inhibition of nitrogenase activity. Although the relief of TlCl-induced inhibition is not to the extent that 1 mM MSX relieved ammonium-induced inhibition, the results do suggest that these two types of inhibitions are at least partially related. It was determined that ammonium causes the greatest degree of nitrogenase inhibition at an constant external pH value of 7.4 over the pH range 6.8 to 8.0 (Figure 19). However, Tl+ inhibited to the greatest extent at pH 7.8 over the same pH range (Figure 19). Although this suggests that the transport processes of these ions are not completely similar, it does not exclude the possiblity that they share a common driving force. It is possible that thallium and ammonium transport in B. alba is driven by the electric membrane

Figure 18. Inhibition of ammonium uptake by thallium chloride. The disappearance of ammonium from the medium of diazotrophic cultures, to which ammonium chloride, final concentration 0.05 mM, was added at time zero, was monitored. TlCl, final concentration 50 µM, was added (○) or not added (●) 2 minutes prior to the addition of ammonium chloride.

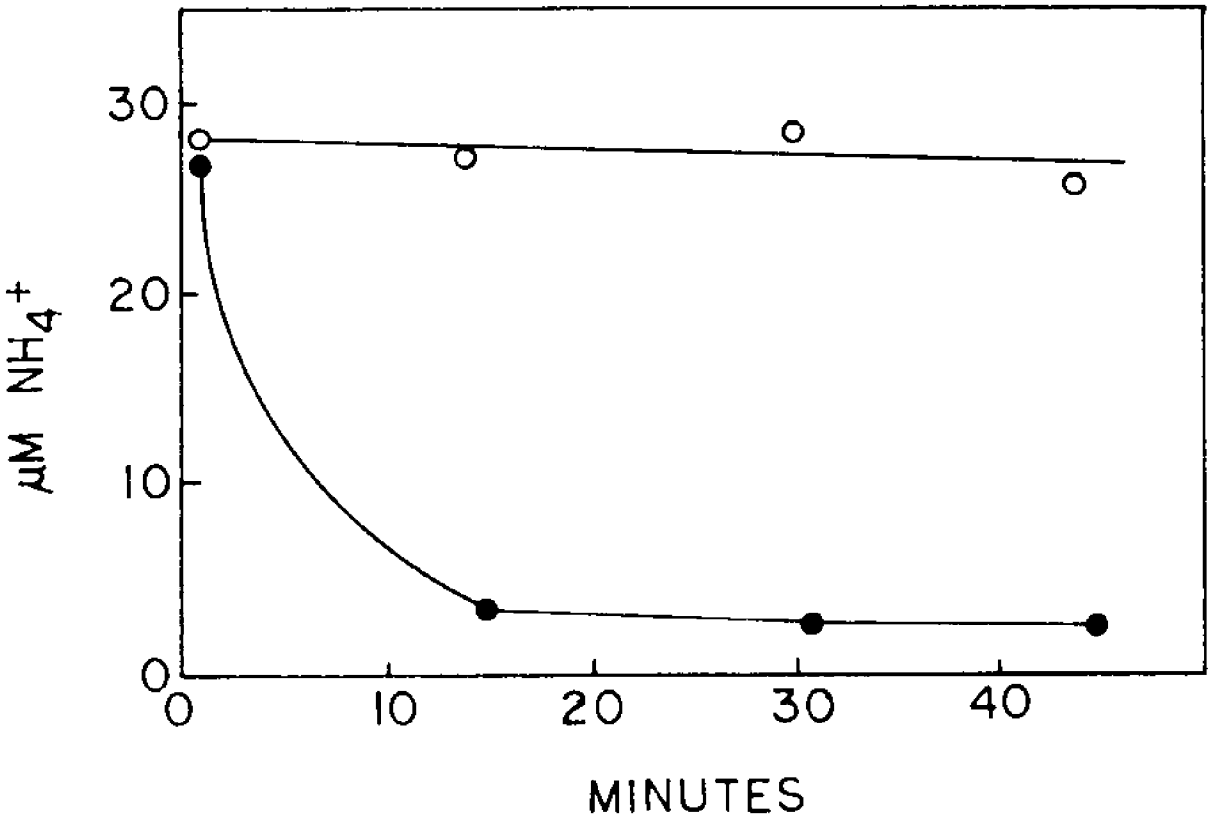

30
20
10
0
0
10
20
30
40
μM NH_4^+
MINUTES

Table 4. Percentage of TlCl-induced inhibition of nitrogenase activity in the presence and absence of MSX.

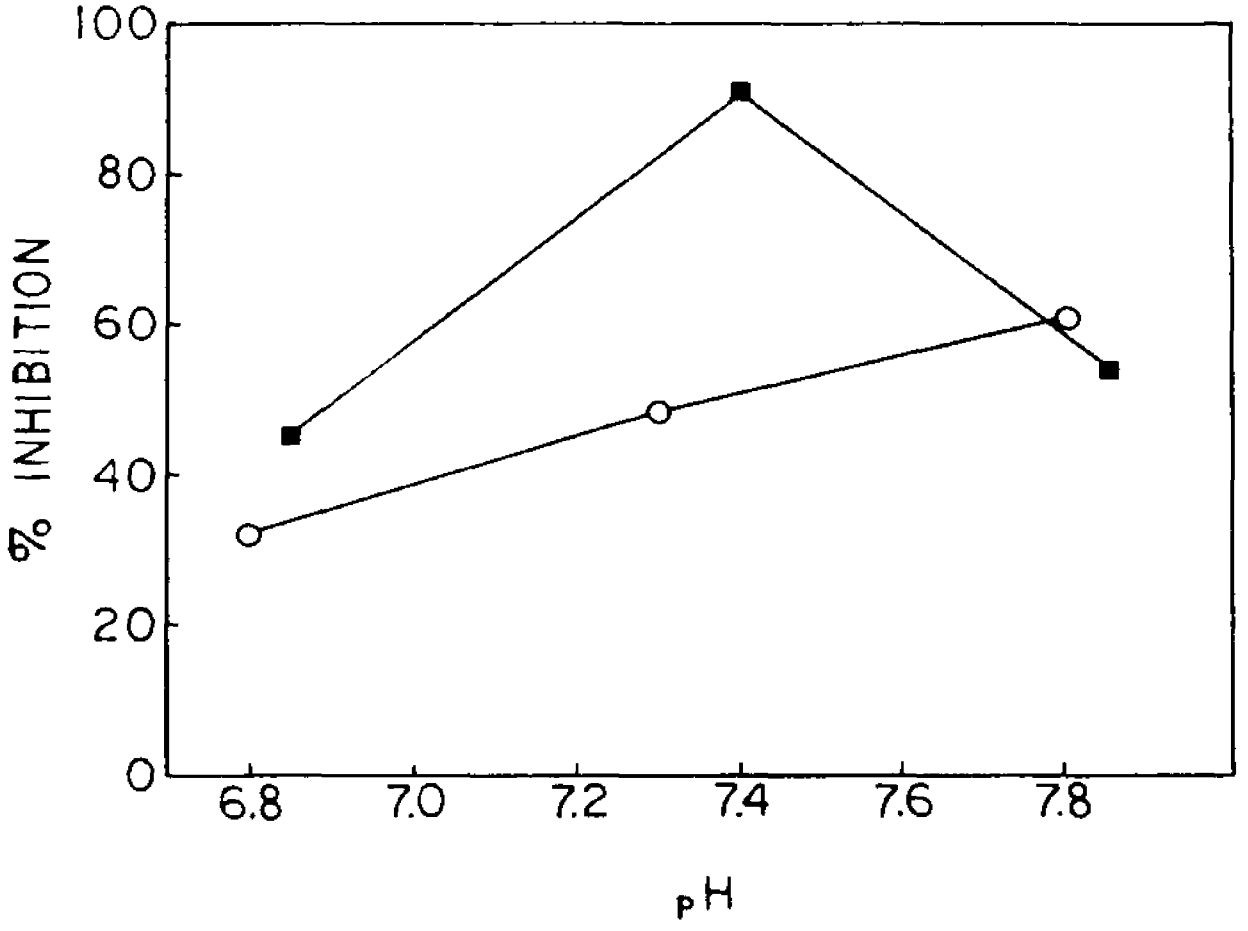
MSX[a]	% Inhibition by TlCl[b]
none	83 +/- 1.4[c]
1 mM	58 +/- 12.0

[a] MSX was added 15 minutes prior to TlCl.

[b] TlCl was added to a final concentration of 50 μM to cell suspensions that were reducing acetylene, as described in methods section.

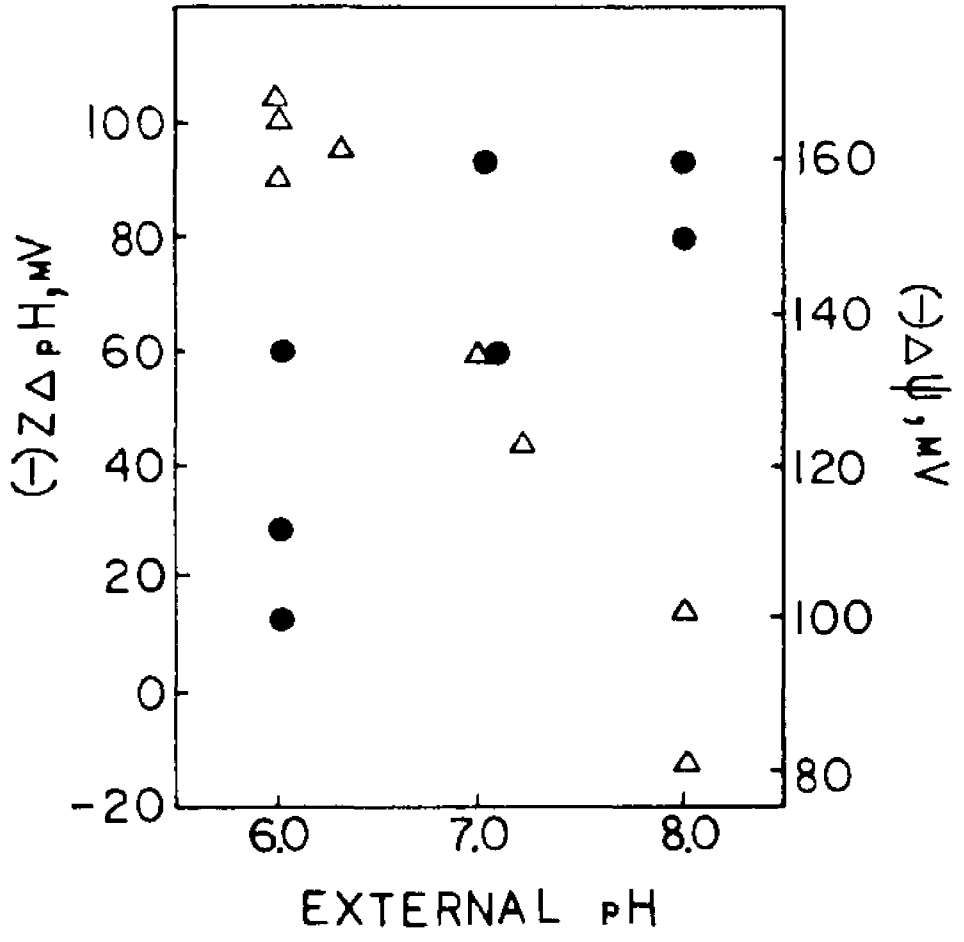
[c] Means and standard deviations were derived from duplicate experiments.

Figure 19. Percentage of ammonium chloride- and thallium chloride-induced inhibition of in vivo nitrogenase activity as a function of external pH. The symbol ■ denotes values for ammonium-induced inhibition and the symbol ○ denotes values for thallium-induced inhibition. External pH was held constant with bis Tris propane buffer as described in Materials and Methods.


100
80
60
40
20
0
% INHIBITION
6.8
7.0
7.2
7.4
7.6
7.8
pH

potential component of the proton motive force. The proton motive force of cell membranes is theoretically composed of two parts: a chemical gradient of protons across the cytoplasmic membrane, called Δ pH, and an electrical gradient contributed to by all charged particles on either side of the membrane, called the membrane potential (15). The specific transport of many ions across the cytoplasmic membrane is controlled by either or both of the components of the proton motive force (15, 28, 31, 43). As external pH increases from pH 6 to pH 8 for a neutrophilic bacterium like E. coli, the membrane potential increases, and thus the ability to transport those ions whose transport processes are controlled by the membrane potential also increases (15, Figure 20). Therefore, the observation that the inhibition of nitrogenase by thallium increases over the pH range 7 to 8 (Figure 19) indicates that thallium transport in B. alba may be driven by the membrane potential. For neutrophilic bacteria, the Δ pH of the cytoplasmic membrane decreases as external pH increases from 6 to 8 (15, Figure 20). Since ammonium inhibition is maximal at pH 7.4, but is lower at pH 6.8 and pH 7.8 (Figure 19), ammonium transport may be related to both Δ pH and the membrane potential. This is an understandable difference between ammonium transport and thallium

Figure 20. Values of proton motive force components as a function of external pH for Escherichia coli. Values for the pH gradient component of the proton motive force ($Z \Delta pH$) are denoted by the symbol $\triangle$, and values for membrane potential component ($\Delta\psi$) are denoted by the symbol ●. Data points were derived from values previously cited (15).


100
80
60
40
20
0
-20
160
140
120
100
80
6.0
7.0
8.0
EXTERNAL pH
(-)ZΔpH, mV
(-)Δψ, mV

transport since the ammonium ion contains a dissociable proton and the thallium ion does not. So, the transport processes of these ions may share a relationship with the membrane potential.

If transport of thallium and ammonium is the phenomenon which causes the inhibition of nitrogenase, then the inhibition may be mediated by transport-related alteration of the proton motive force. Such a mechanism has been postulated for ammonium-induced inhibition of nitrogenase in A. vinelandii (43). There are different ways that an altered proton motive force might cause such an inhibition. One possibility is that changes in pmf (proton motive force) might cause either localized or general changes in cytoplasmic membrane structure due to sudden deviations in surface charge (24). These changes could cause an inhibitory conformational change in nitrogenase if the enzyme had an association with the membrane. A membrane-associated nitrogenase has been proposed for A. vinelandii (24). Such changes in membrane structure could also affect proteins that are involved in electron transport to nitrogenase if they are membrane-associated. Proteins that supply electrons to nitrogenase in A. vinelandii have been found to be associated with the cytoplasmic membrane (42).

In A. vinelandii, the effect that ammonium has on

nitrogenase activity varies with the respiratory state of the cell (41). When the cells are supplied with plenty of oxygen and are in a highly active respiratory state, the effect of ammonium on nitrogenase activity is negligible (41). When the cells are oxygen-limited and thus are in a decreased respiratory state, the addition of ammonium causes nearly complete inhibition of nitrogenase activity (41). Since the respiratory state of a cell is directly related to the magnitude of the proton motive force (15), the different degrees of ammonium-induced inhibition of nitrogenase in A. vinelandii is understandable if pmf is involved in ammonium-induced inhibition. For A. vinelandii incubated under conditions which cause decreased respiration, it was shown that ammonium transport is driven by the cell membrane potential component of the proton motive force, and it was demonstrated that there was a direct correlation between the magnitude of the cell membrane potential and in vivo nitrogenase activity (43). Characteristics of the ammonium-induced inhibition of nitrogenase in B. alba indicate that a similar type of inhibition mechanism may occur in these two bacteria.

Another observation that supports the idea that ammonium-induced inhibition of B. alba nitrogenase activity is related to a change in pmf is that PMS

(phenazine methosulfate), an artificial electron acceptor (51), is capable of inhibiting B. alba nitrogenase activity with kinetics that are similar to the inhibition by ammonium (Figure 21). PMS causes an immediate and incomplete transition in activity (Figure 21). Because of its ability to accept electrons, PMS may inhibit repiratory electron transport, which would affect pmf. PMS might also divert electron flow away from nitrogenase by direct interaction with an electron carrier that functions specifically in electron transport to nitrogenase. 1 mM MSX has no effect on the inhibition that is caused by PMS (Figure 22), which is understandable since the initial site of inhibition for PMS is probably different than that which is involved in ammonium-induced inhibition, even though both PMS and ammonium may affect nitrogenase similarly by producing the same overall effect on the cell.

Since proton motive force has been proposed to be involved in the ammonium-induced inhibition it is possible that by decreasing the pmf, the ATP content of the cells is affected. This is another possibility for the overall mechanism by which ammonium, and possibly TlCl and PMS, affects nitrogenase activity, since nitrogenase requires a large amount of ATP for its reaction (24). In order to test this, the effects of

Figure 21. Immediate effect of phenazine methosulfate (PMS) on in vivo acetylene reduction. PMS was added at the time indicated by the arrow to one of two replicate acetylene-reducing cell suspensions. The symbol ● denotes the culture which received PMS at a final concentration of 0.1 mM, and the symbol △ denotes the culture which did not receive PMS.

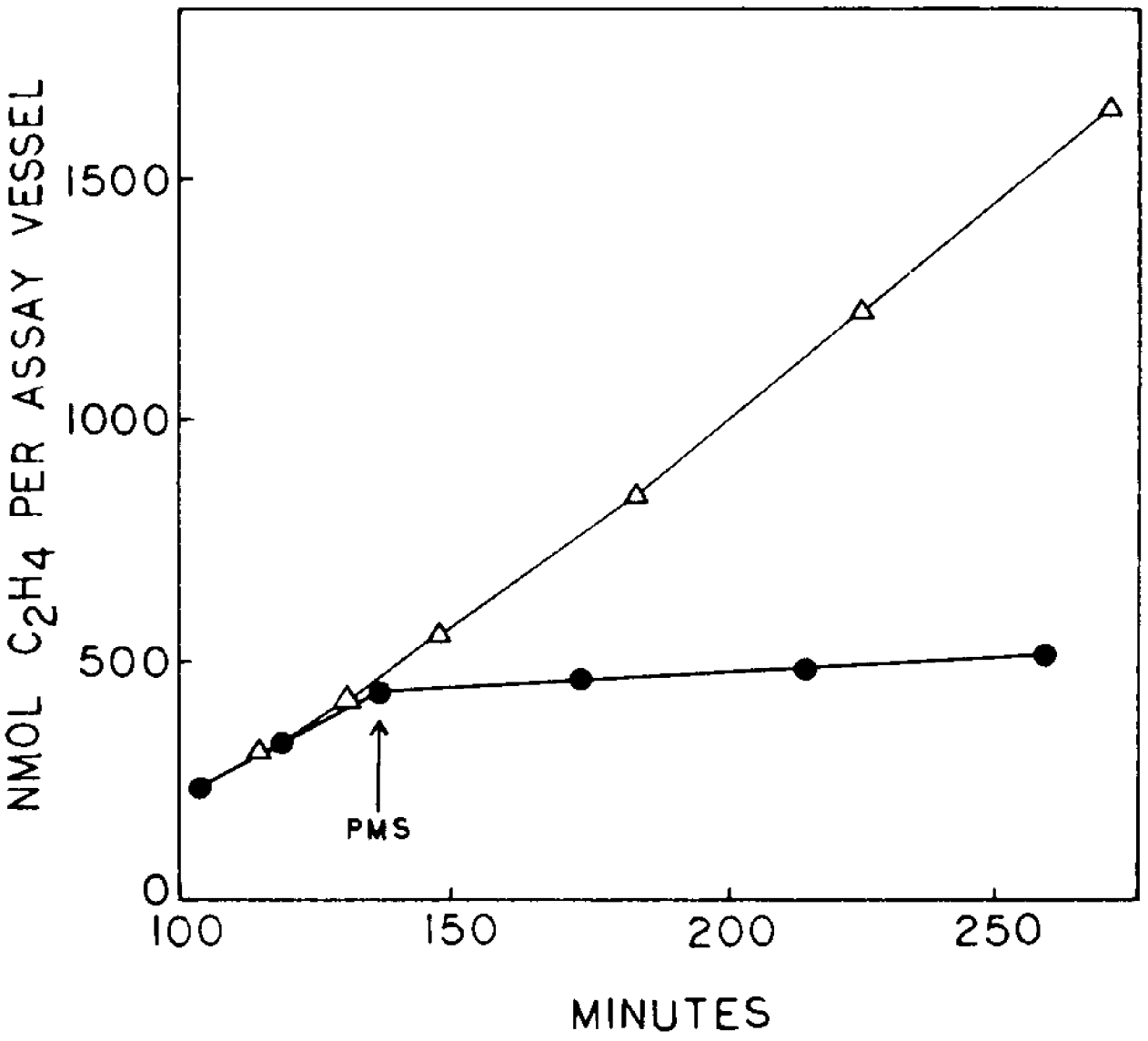
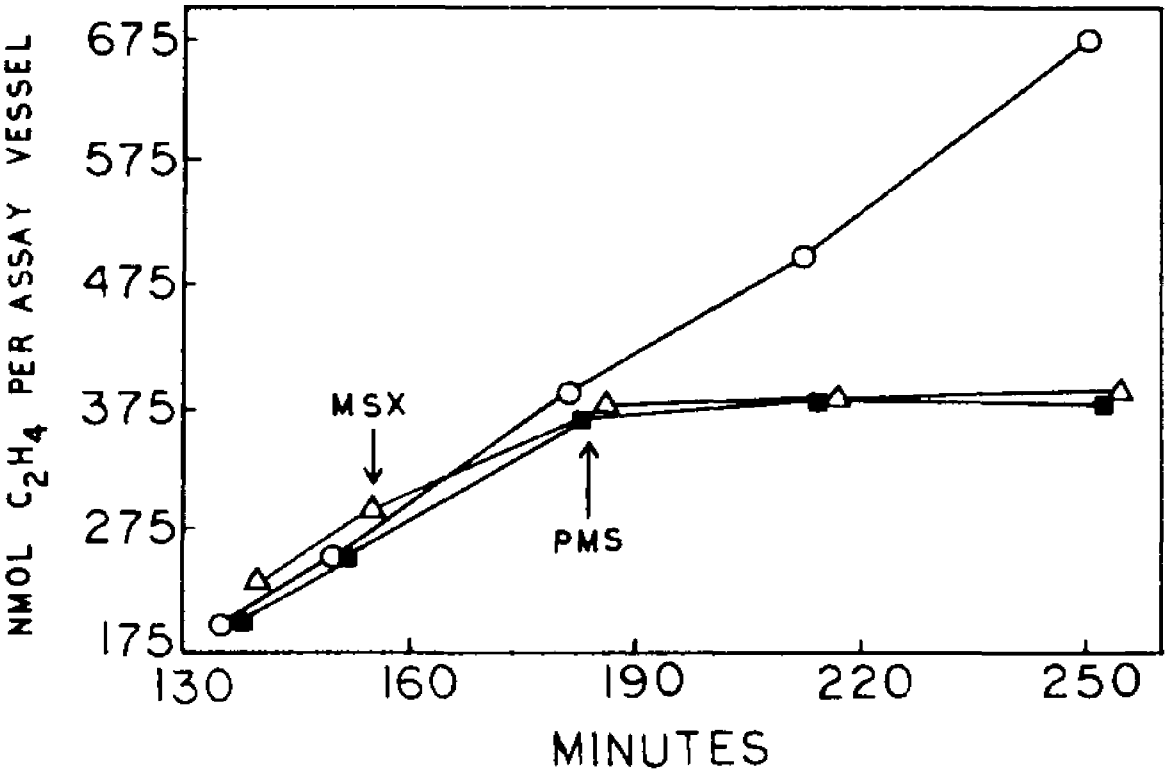

NMOL C_2H_4 PER ASSAY VESSEL
1500
1000
500
0
100
150
200
250
MINUTES
PMS

Figure 22. Effect of 1 mM MSX on phenazine methosulfate (PMS) inhibition of nitrogenase activity. Acetylene-reducing cultures received the following additions: ○, none; ■, PMS; △, MSX, then PMS. An arrow indicates the time at which MSX, final concentration 1 mM, was added. Another arrow indicates the time at which PMS, final concentration 0.1 mM, was added.


NMOL C_2H_4 PER ASSAY VESSEL
675
575
475
375
275
175
130
160
190
220
250
MINUTES
MSX
PMS

DCCD (dicyclohexylcarbodiimide), an inhibitor of the membrane-associated, proton-translocating ATPase found in E. coli and other bacteria (15), and arsenate, which typically inhibits oxidative phosphorylation (51), were tested. Neither compound had an effect on nitrogenase activity (Figures 23 and 24). However, when the ability of these compounds to affect the growth of B. alba was tested, it was found that neither chemical inhibits growth (Figure 25). Apparently, B. alba is resistant to the toxic effects of these compounds. This is interesting in light of the fact that arsenate resistance in some bacteria is plasmid-encoded (60), and B. alba B18LD contains 3 plasmids (see Part I). Because of these results, a role for ATP limitation in the mechanism of ammonium-induced inhibition of nitrogenase can not be ruled out.

The possibility remains that ammonium directly interacts with B. alba nitrogenase in a feedback type of inhibition. This is unlikely for different reasons. One is that this type of direct inhibition has never been observed for a nitrogenase in another organism, and nitrogenase enzymes among most bacteria appear to have highly conserved sequences (72, 78). Another reason is that nitrate and nitrite do not inhibit B. alba nitrogenase activity. Since B. alba metabolism of these

Figure 23. Immediate effect of sodium arsenate on in vivo acetylene reduction. Arsenate was added at the time indicated by the arrow to one of two replicate acetylene-reducing cell suspensions. The symbol ○ denotes the culture which received arsenate at a final concentration of 30 mM, and the symbol ■ denotes the culture which did not receive arsenate.

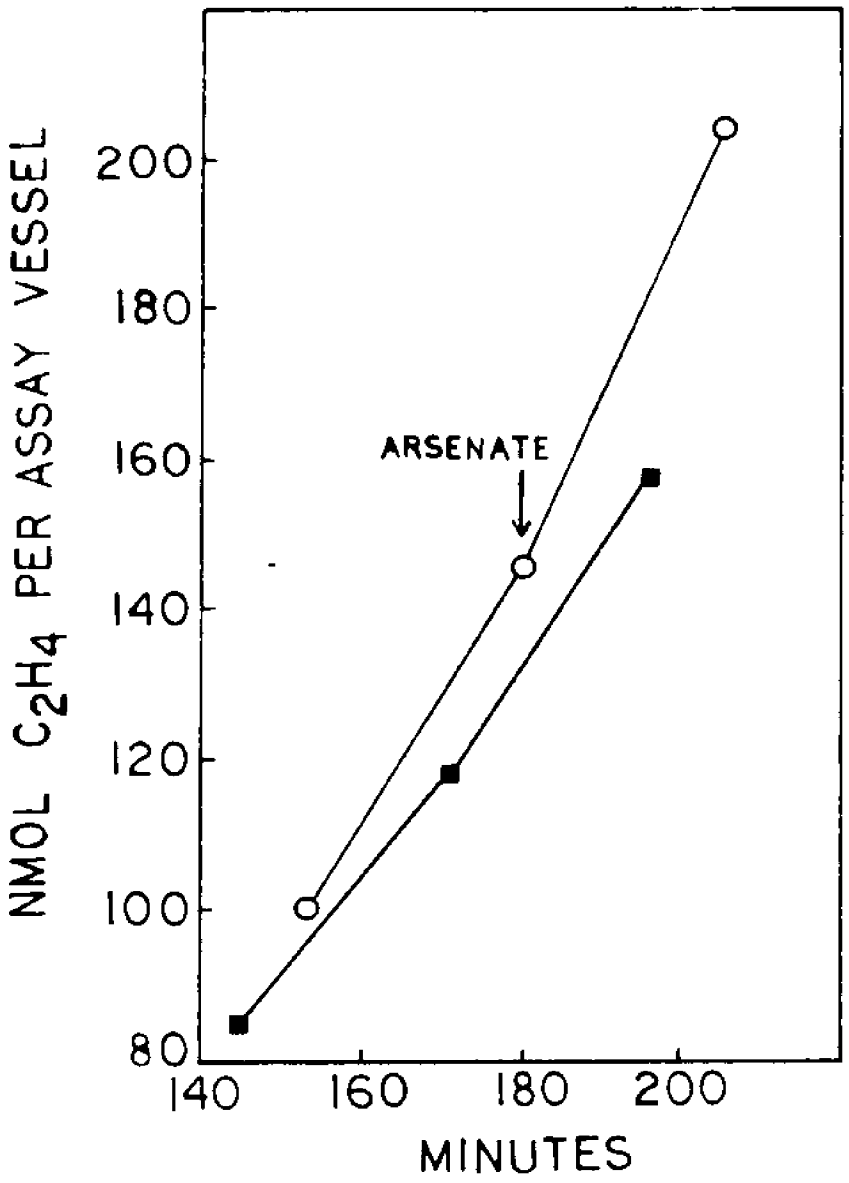

NMOL C_2H_4 PER ASSAY VESSEL
200
180
160
140
120
100
80
ARSENATE
140
160
180
200
MINUTES

Figure 24. Immediate effect of dicyclohexylcarbodiimide (DCCD) on in vivo acetylene reduction. DCCD was added at the time indicated by the arrow to one of two duplicate acetylene-reducing cell suspensions. The symbol ▲ denotes the culture which received DCCD at a final concentration of 1 mM, and the symbol ○ denotes the culture which did not receive DCCD.

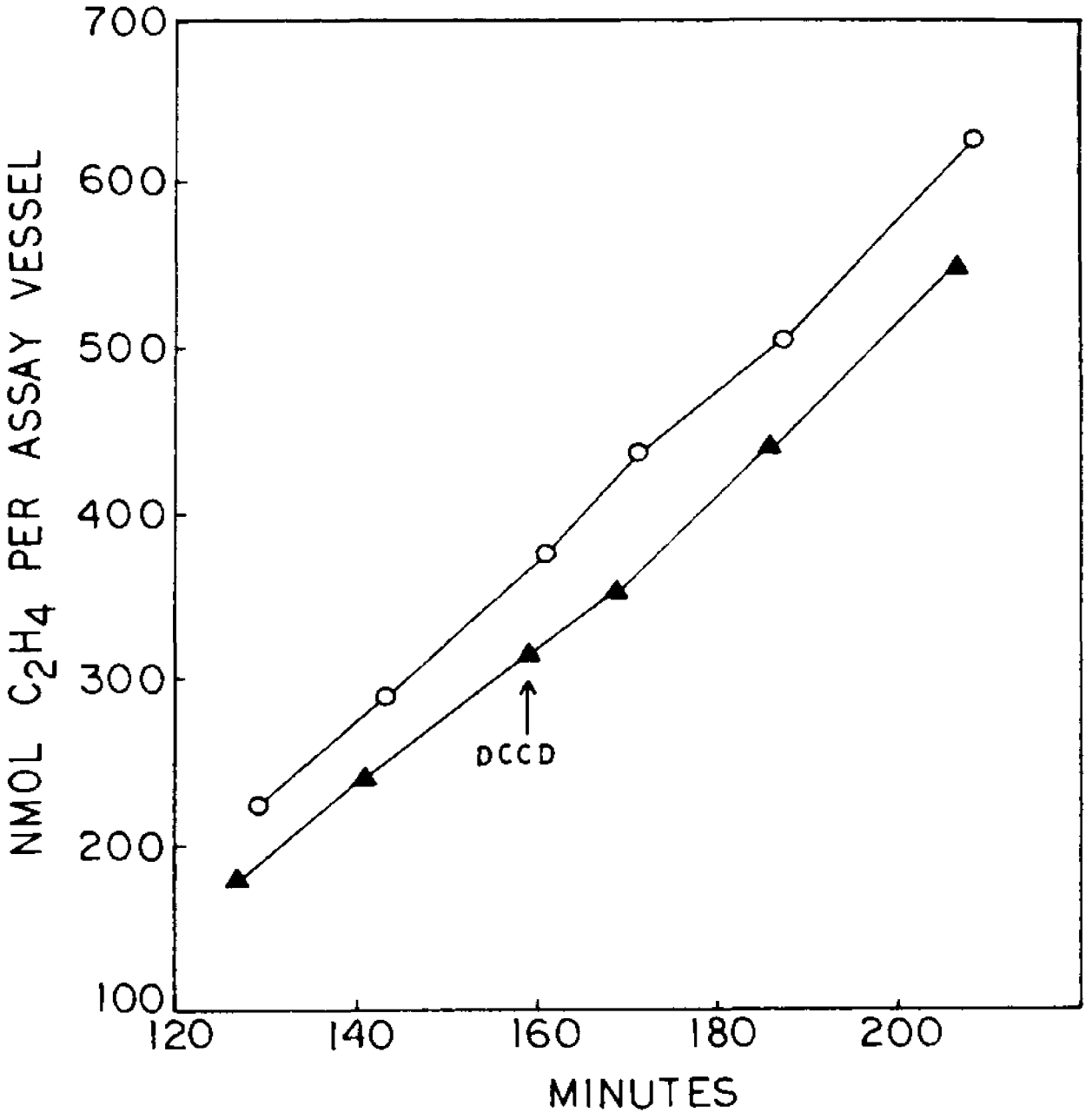
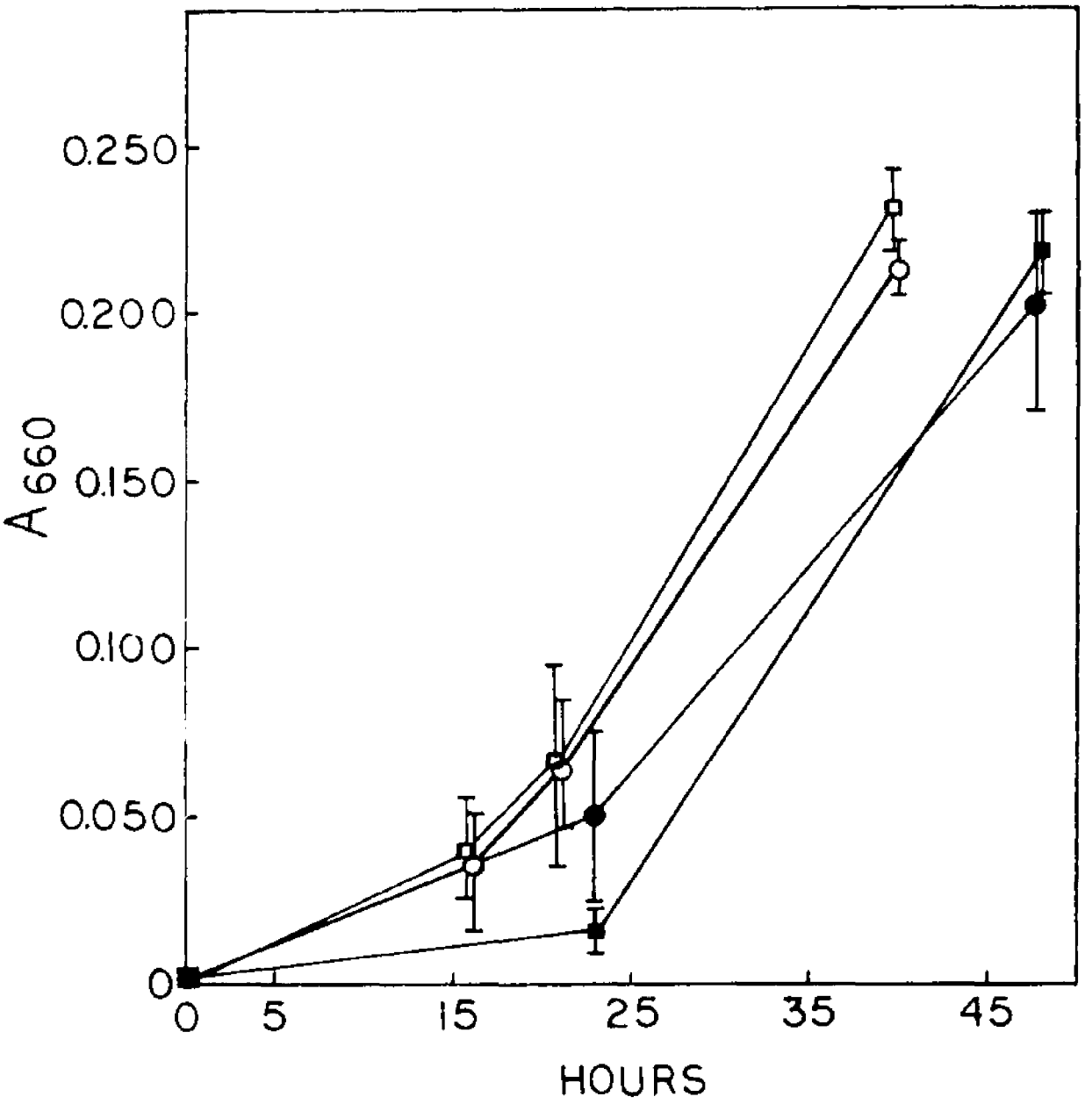

700
600
500
400
300
200
100
NMOL C_2H_4 PER ASSAY VESSEL
120
140
160
180
200
MINUTES
DCCD

Figure 25. Growth of B. alba cells in the presence of arsenate and dicyclohexylcarbodiimide (DCCD). Cells were inoculated into shaker flasks containing AC medium and the following additions: ○, none; □, sodium arsenate; ■, DCCD (dissolved in 1 ml acetone); ●, 1 ml acetone. Arsenate and DCCD were added to final concentrations of 10 mM and 0.1 mM, respectively.


0.250
0.200
0.150
0.100
0.050
0
A660
0
5
15
25
35
45
HOURS

ions is assimilatory (100), ammonia is an intermediate in their assimilation, but neither nitrate nor nitrite has any direct effect on nitrogenase activity (Table 2). An aditional observation that does not support a direct inhibition of nitrogenase is that the inhibition is immediate and incomplete at several of the concentrations of ammonium tested (Table 3). These kinetics suggest that an immediate effect is made on the cell as soon as ammonium is added to the medium, and since the cell membrane is probably encountered almost immediately by the ammonium ions, then an alteration in the character of the cell membrane, i. e. pmf, is a more likely possiblity for the mechanism of inhibition. Nevertheless, attempts were made to produce cell-free crude nitrogenase extracts in order to test for direct inhibition of nitrogenase by ammonium. Cell lysis was attempted in one case by the osmotic lysis method that has been used successfully with A. vinelandii (B. Hales, personal communication), but this method caused lysis of less than 20% of the B. alba cells, and nitrogenase activity was absent in the resultant cell-free extracts. In another experiment, a combination of freeze-thawing the cells with liquid nitrogen, lysozyme treatment, and sonication achieved almost 100% lysis of the cells, but no nitrogenase activity was observed for the extracts, and it was found

after the assay that the final pH of the lysate was 4.2, which explains the lack of activity. The extremely lowered pH was probably caused by the low concentration of buffer in the cell suspension (25 mM Tris, pH 7.5). After the experiment, the pH of the extract was raised to 7.1 with 1 M Tris, pH 7.5, and it was found that the buffer concentration should have been 150 mM Tris in order to maintain adequate pH control.

In conclusion, the following observations and proposals were made about the physiology of nitrogen fixation in B. alba B18LD:

1. B. alba is extremely microaerophilic with respect to nitrogenase activity.

2. Nitrogenase activity shows a broad pH optimum that is indicative of a neutrophile, and a narrow temperature optimum at 29°C that is indicative of a mesophile, and of most nitrogenases.

3. Ammonium assimilation products are not repressive or inhibitory to nitrogenase activity, however nitrate, nitrite, and chloramphenicol block the induction of activity, and thus B. alba nitrogenase is not a constitutive enzyme and B. alba nif genes are probably subject to a repressive type of regulation.

4. Ammonium causes an immediate short-term inhibition of nitrogenase activity, which is probably mediated by

the effect of ammonium transport on the proton motive force.

Part III. Attempts to locate and clone *Beggiatoa alba* B18LD nitrogenase genes.

INTRODUCTION

From those nitrogen-fixing genetic systems that have been well studied, it can be concluded that the molecular biology of nitrogen fixation is complex. This is exemplified by Klebsiella pneumoniae, a bacteria whose DNA contains approximately 20 different nif (nitrogen fixation) genes (7, 72, 97). 13 of these are required for efficient nitrogen fixation (72, 97). These genes encode the structural subunits of nitrogenase and also proteins with various other functions such as electron transport to nitrogenase, synthesis of nitrogenase-related cofactors, processing of the dinitrogenase reductase component of nitrogenase, and genetic regulation of nitrogen fixation (7, 72, 97). Other types of nif genes which have been found in other nitrogen-fixing bacteria include alternate nitrogenase structural genes in Azotobacter chroococcum and Azotobacter vinelandii (9, 25, 76). These encode alternate nitrogenases which use vanadium or iron as alternate cofactor metals to molybdenum (9, 25, 76).

The nitrogenase structural genes for Klebsiella pneumoniae have been cloned and are designated as nifH, which encodes the sole subunit of dinitrogenase reductase, nifD, which encodes the alpha subunit of

dinitrogenase, and nifK, which encodes the beta subunit of dinitrogenase (7). These genes show considerable sequence homology with nif structural genes from other bacteria (78) and this property has been exploited to clone the nifH, nifD, and nifK genes from such diverse nitrogen-fixing bacteria as Azotobacter vinelandii (6), Rhizobium meliloti (78), Thiobacillus ferrooxidans (73), and Methanococcus voltae (82).

The purpose of this study was to clone nitrogenase structural genes from Beggiatoa alba B18LD. The strategy was to first create a library of B. alba DNA fragments by insertion of such fragments into a plasmid vector and propagation of the recombinant plasmids in E. coli, and then to screen these recombinant strains for those which contained B. alba nif genes. Specifically, random AluI restriction endonuclease fragments of B. alba DNA were inserted into the EcoRV restriction enzyme site of plasmid pBR322, which encodes tetracycline and ampicillin resistance (55). Insertion into the EcoRV site interrupts the tetracycline resistance gene (55). Such recombinant plasmids were used to transform E. coli NM522 (20), and clones were selected on the basis of ampicillin resistance and tetracycline sensitivity. The plan was then to screen

these recombinant strains for DNA encoding B. alba nitrogenase genes by employing a Klebsiella pneumoniae nifHDK DNA probe that would supposedly hybridize to recombinant colonies which contained B. alba nif DNA. Unfortunately, the Klebsiella pneumoniae probe did not hybridize significantly with restriction enzyme digests of B. alba DNA and therefore did not appear to be usable as a screening probe for recombinants. Various attempts were made to resolve the problem of weak hybridization, but a strong hybridization reaction was never observed. So, none of the recombinant strains have been screened yet for B. alba nif genes.

Although the attempts to clone B. alba nif genes were unsuccessful, two interesting accomplishments came out of the study. One is the observation that B. alba B18LD DNA is refractory to digestion by several restriction endonucleases. This property has been observed with other bacteria and usually indicates that one or more of the nitrogenous bases of the DNA is modified (1, 2, 44, 68). Another beneficial result that was an offshoot of this study was the development of a new technique for purifying electrophoretically-separated DNA from agarose gels. The procedure is a modification of the "freeze-squeeze" method (96), and is

an improvement over this method as well as all others reported in that it is rapid, easily reproducible, and gives good yields of DNA.

MATERIALS AND METHODS

Purification of B. alba total DNA.

Cells were cultivated in 1 liter of modified AC broth in a 2 liter flask as described in Part II, to a optical density of approximately 0.250 at 660 nm. The methods used for the lysis of cells and purification of DNA have been described previously (32, 59). The cells were centrifuged, resuspended in 40 ml TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) which contained 80 mg of lysozyme, and transferred to a 125 ml flask. The mixture was incubated at 34°C for 15 minutes. 2.6 ml 20% (w/v) aqueous sodium dodecyl sulfate was added, and the mixture was incubated at 57°C for 15 minutes. 13.2 ml 5 M sodium perchlorate was mixed into the lysate, and the mixture was transferred to a 100 ml ground glass-stoppered graduated cylinder. 32 ml of chloroform-isoamyl alcohol, 24:1 (v/v) was added. The cylinder was sealed and shaken gently for 30 minutes. The mixture was transferred to a 250 ml centrifuge tube and centrifuged at 12,000 x g for 10 minutes. 60 ml of the aqueous upper layer was transferred to another glass graduated cylinder, and 30 ml of chloroform-isoamyl alcohol was added and the mixture was shaken for 30 minutes, then centrifuged. 44 ml of the aqueous layer was transferred to a 250 ml

centrifuge tube. 4.6 ml 3 M sodium acetate was mixed in and then 101 ml cold 95% ethanol was added and mixed in. The mixture was incubated at -20°C overnight, then centrifuged at 7000 x g for 30 minutes. The ethanol was poured off, and the pellet was dried with a stream of sterile air, then dissolved in 10 ml TE buffer. 357 µl of a ribonuclease solution was added that was prepared by dissolving 14 mg of ribonuclease A in 10 ml of water, then heating the solution in boiling water for 10 minutes. The mixture was incubated at 37°C for 30 minutes, then transferred to a glass graduated cylinder. 5 ml of chloroform-isoamyl alcohol was added, and the mixture was shaken for 15 minutes, then centrifuged at 12,000 x g, 15 minutes. 7.5 ml of the upper aqueous layer was transferred to a 30 ml centrifuge tube, and 0.75 ml 3 M sodium acetate and 16.5 ml cold 95% ethanol were mixed in. The tube was incubated at -20°C for 1 hour, then centrifuged at 12,000 x g 10 minutes. The supernatant was discarded, and the pellet was air-dried and resuspended in 5 ml of TE buffer. Ethanol precipitation was repeated again with 0.5 ml 3 M sodium acetate and 11 ml ethanol, and the pellet was dissolved in 3 ml TE buffer. Precipitation was performed a third time with 0.3 ml sodium acetate and 6.6 ml ethanol, and the final pellet was dissolved in 5 ml TE buffer. The

total yield of DNA was 0.450 mg as determined by ultraviolet spectrophotometric analysis of a 50 µl portion at 260 nm.

Transfer of DNA to nitrocellulose membranes. 75 µl (6.75 µg) of purified B. alba total DNA was ethanol precipitated in a microfuge tube and the pellet was suspended in 500 µl of 70% ethanol at room temperature by vortexing. The tube was centrifuged 5 minutes, the ethanol was discarded, and the pellet was air-dried and resuspended in 22.5 µl of sterile deionized water. 2.5 µl of 10x HindIII restriction endonuclease buffer was added to the tube. The buffer was prepared according to the specifications of the enzyme manufacturer (Sigma Chemicals). 20 U of HindIII was then added to the tube, which was then incubated at 37°C for 1 hour. The digest was mixed with gel-loading buffer, loaded onto a 60 ml 1% (w/v) horizontal agarose gel, along with other DNA samples, and electrophoresed at 50 V for 3 hours, as described in detail in Part I. The gel was stained with ethidium bromide, and photographed as described previously. A 4.2 x 6.2 cm portion of the gel, that contained the DNA of interest, was bathed, with gentle agitation, in 150 ml of 0.25 M HCl for 10 minutes. The gel was then bathed, with agitation, in 150 ml water for 2 minutes, then in 150 ml of 1.5 M NaCl, 0.5 M NaOH

for 1 hour. The gel was bathed, with agitation, in 150 ml 1 M Tris, pH 8.0, 1.5 M NaCl for 1 hour. The DNA was then transferred from the gel to a nitrocellulose membrane by capillary diffusion, for 16 hours exactly as described on pages 383 to 385 in Maniatis et al. (55) . After transfer, the membrane was air-dried 30 minutes, then baked for 2 hours in a vaccum oven evacuated to 28.5 inches Hg at 80°C. The filter was stored at room temperature, sealed in a plastic petri dish with Parafilm.

Purification of probe DNA. The probe used is a 4.4 Mdal EcoRI DNA fragment from Klebsiella pneumonia that contains the complete structural genes for the nitrogenase enzyme complex, nifH, nifD, and nifK (7). This fragment is contained in a 6.9 Mdal plasmid, called pSA30, that is carried in Escherichia coli strain HB101 (R. Saganich, personal communication).

E. coli HB101/pSA30 was cultivated overnight in 25 ml LBT medium shaking in a 50 ml flask at 250 rpm, 37°C, overnight, to an optical density of 0.959 at 600 nm. LBT medium is prepared by mixing, per liter, 10 g Bacto-tryptone, 5 g yeast extract, and 10 g NaCl, then adjusting the pH to 7.5 (55). This solution is autoclaved and cooled to room temperature. To this is

added 1.2 ml of a filter-sterilized tetracycline solution prepared by dissolving 12.5 mg of tetracycline per ml of 50% (v/v) ethanol (55). The culture was centrifuged at 7000 x g for 7 minutes and the cells were resuspended in 8 ml TE buffer. This was transferred to a 100 ml beaker. 16 ml of Kado-Liu lysing buffer (see Part I, Materials and Methods) was mixed in, and the beaker was incubated at 56°C for 72 minutes. The lysate was then cooled to room temperature and the pH was adjusted to 8.4 with approximately 4 ml of 1 M Tris-Cl, pH 7.5. 56 ml of chloroform-isoamyl alcohol was added and the mixture was gently shaken for 35 minutes, then centrifuged at 12,000 x g for 30 minutes. 25 ml of the aqueous phase was transferred to a 250 ml centrifuge tube, and the DNA was precipitated with 2.5 ml of 3 M sodium acetate and 55 ml of 95% ethanol overnight at -12°C. The pellet was dissolved in 10 ml of TE buffer, and the resulting pSA30 plasmid DNA solution was stored at 4°C.

750 µl of the pSA30 solution was precipitated with ethanol and the pellet was washed with 2 ml 70% ethanol. The DNA was then dissolved in 250 µl of EcoRI restriction enzyme buffer, 50 U of EcoRI was added, and the mixture was incubated at 37°C for 1.5 hours. The entire digest was then mixed with gel-loading buffer and loaded onto a horizontal 0.5% (w/v) 60 ml agarose gel, into a large

well with dimensions 50x1x7mm. The gel was electrophoresed at 50V for 2.5 hours, stained with ethidium bromide, and washed in water. A trough was cut in front of the 4.4 Mdal EcoRI DNA band, and a slit was cut in back of the band. The slit and the trough were then lined with dialysis membrane (103). The trough was filled with 2.5 ml of electrophoresis buffer (103), and the DNA was electroeluted into the trough at 25 milliamps for 30 minutes. The charge of the electrophoresis chamber was then reversed for 1 minute (103), and the trough buffer was transferred to a glass vial. The trough was washed with another 2.5 ml of buffer and this was added to the glass vial. The electroeluted DNA was then purified with an Elutip-D column exactly as described in the instruction manual prepared by the manufacturer (Schleicher and Schuell). The final volume of the nifHDK DNA solution was 0.550 ml, which contained 2.8 µg of DNA. 0.4 ml (2 µg of DNA) of this solution was precipitated with ethanol and the pellet was dissolved in 50 µl of TE buffer. The DNA was then non-radioactively labelled by chemical sulfonation with a Chemiprobe kit exactly according to the instructions supplied by the manufacturer (FMC BioProducts, Rockland, Maine). The treatment basically involves exposing the DNA to methoxyamine and bisulfite, which results in the

formation of sulfone groups on the cytosines of the DNA.

Hybridization of filter-bound DNA to probe DNA and detection of probe DNA. A 6 5x4.5 cm nitrocellulose filter containing B. alba DNA that was prepared as described above, was bathed in 100 ml 6x SSC buffer for 2 minutes (55). 6x SSC is prepared from a stock solution of 20x SSC, which is made by mixing 175.3 g of NaCl and 88.2 g of sodium citrate in 800 ml water, adjusting the pH to 7.0, then bringing the volume to 1 liter (55). The wet filter was then placed in a Dazey Seal-a-meal bag and prehybridization solution pre-warmed to 68°C was added. The bag was sealed shut and then incubated at 68°C for 5 hours (55). Prehybridization solution contained 879 µl of water, 450 µl of 20x SSC, 6 µl of 25% SDS, 150 µl of 50x Denhardt's solution, and 15 µl of denatured salmon sperm DNA (stock solution, 10 mg/ml water). 50x Denhardt' solution contains, per liter of water: 10 g Ficoll 400, 10 g of polyvinylpyrrolidone, and 10 g of bovine serum albumin (55). After prehybridization, the bag was cut open, the prehybridization solution was squeezed out, and hybridization solution was added. The bag was resealed and incubated at 68°C for 17 hours (55). Hybridization solution contained: 794 µl of water, 450 µl of 20x SSC, 6 µl of 25% SDS, 150 µl of 50x Denhardt's solution, and

162 µl of sulfonated nifHDK solution (2 µg DNA). After hybridization, the filter was removed from the bag and washed in 90 ml of 2x SSC, 0.1% SDS for 20 minutes at room temperature, then washed in 90 ml of 0.1x SSC, 0.1% SDS for 30 minutes at room temperature. The membrane was treated for probe visualization exactly according to the instructions provided for the Chemiprobe kit. The visualization procedure basically involves binding the probe DNA with monoclonal antibodies that specifically react with sulfonated DNA, then binding these antibodies with anti-antibodies that are conjugated to alkaline phosphatase, and finally detecting the location of alkaline phosphatase on the filter with a chromogenic substrate that produces a purple coloration whereever probe DNA has hybridized to filter-bound DNA.

Cloning of B. alba DNA. To prepare vector DNA, 1 µg of pBR322 (Sigma Chemicals) in 400 µl of TE buffer was mixed in a microfuge tube with 48 µl of water, 50 µl of 10x EcoRV buffer and 20 U of EcoRV restriction endonuclease. This was incubated for 3.5 hours at 37° C. The reaction was terminated by heating at 65°C for 10 minutes. 9 µl of 57 mM $ZnCl_2$ and 5 µl bacterial alkaline phosphatase (0.8 U) were added (55). This was incubated for 1 hour at 60°C. 2 µl of 25% SDS and 15 µl of proteinase K (stock solution, 100 µg/ml water) were

added, and the mixture was incubated at 37°C for 1 hour. 250 µl of chloroform-isoamyl alcohol was added, and this was gently shaken for 15 minutes, then centrifuged for 5 minutes, and the aqueous upper layer was transferred to another microfuge tube. The chloroform treatment was repeated three more times. The final aqueous solution was precipitated with sodium acetate and ethanol overnight at -20°C. The DNA pellet was resuspended in 100 µl of TE buffer. This contained 500 ng of DNA, as determined by electrophoresing a small portion in the same gel as known quantities of linearized pBR322, and comparing the band intensities after staining with ethidium bromide (55).

To prepare B. alba DNA for ligation to the vector DNA, 183 µg of purified total DNA was ethanol precipitated and the pellet was dissolved in 229 µl of water. 24 µl of 10x AluI buffer and 11 µl (11 U) of AluI restriction enzyme were added and the mixture was incubated for 2 hours at 37°C. This allowed only partial digestion of the DNA. The reaction was terminated by heating at 65°C for 10 minutes. The digest was mixed with gel-loading buffer and loaded into a large electrophoresis well of a 0.5% (w/v) agarose gel as described above for the purification of probe DNA. A small sample of size marker DNA (lambda HindIII digest)

was loaded into a smaller well next to the large well. Electrophoresis was performed at 50 V for 2.5 hours. The size marker lane was cut away from the rest of the gel and stained with ethidium bromide. This lane was used to indicate which portion of the unstained gel piece corresponded to the size range of DNA from 3 to 4.2 Mdal. A slit was cut in back of this size DNA, and a trough was cut in front. The trough and slit were lined with dialysis membrane, and 1.8 ml electrophoresis buffer was added to the trough. DNA was electroeluted into the trough at 50 V for 30 minutes. The current was reversed for 2 minutes, and the trough buffer was transferred to microfuge tubes. The tubes were centrifuged for 10 minutes to pellet small pieces of gel. The supernatant was treated once with chloroform-isoamyl alcohol as described above, and the final aqueous solution was precipitated with ethanol overnight at -20°C. The pellet was washed in 70% ethanol, and the final DNA pellet was dissolved in 400 µl of TE buffer. The solution contained 2.2 µg of DNA as determined by ultraviolet spectrophotometric analysis of a small portion.

The following ligation mixture was prepared: 37 µl of water, 5 µl of 10x ligation buffer, 2 µl of *Eco*RV-digested, phosphatase-treated pBR322, 10.5 µl of

AluI-digested B. alba DNA, and 5 µl (5 U) of T4 DNA ligase (Bethesda Research Labs). 10x ligation buffer contains 0.66 M Tris-Cl, pH 7.5, 50 mM $MgCl_2$, 50 mM dithiothreitol, and 10 mM ATP (55). The mixture was incubated at 25°C for 25 hours. The ligase mixture was used to transform E. coli NM522 (20) in the following manner. E. coli NM522 was cultivated overnight at 37°C, 200 rpm, in LB broth. LB has a similar composition to LBT described above except that tetracycline is omitted. 1 ml of culture was transferred to 100 mls of LB in a 500 ml flask, and this was shaken at the same temperature and speed for 70 minutes, at which time the optical density was 0.191 at 550 nm. 18 ml of the culture was transferred to a 30 ml centrifuge tube and chilled on ice for 10 minutes. The cells were centrifuged at 7000 x g, 5 minutes, and resuspended in 9 ml of cold 50 mM $CaCl_2$, 10 mM Tris-Cl, pH 8.0. The tube was placed on ice for 15 minutes. The cells were centrifuged, and resuspended in 1.2 ml 50 mM $CaCl_2$, 10 mM Tris-Cl, pH 8.0. 200 µl of cell suspension was placed in a cold microfuge tube, and incubated at 4°C for 24 hours. The entire ligation mixture was added to the tube and mixed in, then the tube was cooled on ice for 30 minutes. The tube was placed in a 42°C water bath for 2 minutes. 1 ml of LB broth was added and the tube was

incubated at 37°C for 1 hour. Five 250 µl aliquots were placed into separate test tubes containing 3 ml LBA top agar (LBA medium plus 0.7% agar, w/v) at 47°C. LBA is the same as LBT medium except that ampicillin is included, instead of tetracycline, at a final concentration of 40 µg/ml. The tubes of top agar were immediately poured onto the surface of LBA agar in five separate petri dishes. The top agar was allowed to solidify for 1 hour, then the plates were inverted and incubated at 37°C for 12 hours and then at 27°C for 12 hours. Each ampicillin resistant colony was transferred with a toothpick to a petri dish containing LBA agar and to a petri dish containing LBT agar. Colonies which were sensitive to tetracycline were presumed to consist of cells which contained vector molecules that had B. alba AluI inserts in the EcoRV site of the tetracycline gene of pBR322. To test this, 5 of these colonies were grown up overnight in LBA, and plasmid DNA was isolated from each according to the Kado-Liu method described above. 3 of the 5 bacterial strains contained a single plasmid type approximately 6.4 Mdal in size. The other two strains did not contain plasmid DNA. The ligation and transformation procedures were repeated on different occasions to produce a total of 180 ampicillin resistant, tetracycline strains, most

of which were probably recombinant organisms.

Restriction enzyme digestion of B. alba DNA. All restriction endonuclease reactions were performed with enzymes purchased from Sigma Chemicals in the presence of assay buffers prepared specifically for each enzyme according to the supplier. For some enzymes which were unable to digest B. alba DNA, the integrity of the DNA prep and the restriction enzyme was tested by one or more of three experiments: (1) the enzyme was used to digest preps of either plasmid pBR322 (55) or pLB1 (6), (2) the enzyme was used to digest pBR322 mixed with the B. alba DNA preparation, and (3) E. coli cells containing pBR322 and B. alba cells were mixed and total DNA was purified from the mixture of cells by the same procedure that is described above for the purification of B. alba total DNA, then the restriction enzyme was used to digest the mixture of DNA, and its ability to digest pBR322 was analyzed.

New technique for the purification of DNA from agarose gels. Because the electroelution method of purifying DNA from agarose gels described above is tedious, a rapid new method was devised and tested that is a modification of the previously reported "freeze-squeeze" method. Duplicate DNA samples were loaded onto a 0.5% (w/v) 70 ml

horizontal agarose gel (well dimensions, 6x1x8mm). The amounts of DNA loaded onto the gel for each type of DNA were as follows: B. alba total DNA, 7.3 µg; lambda HindIII digest, 2.8 µg; pBR322, 75 ng. The agarose was SeaKem LE (FMC Bioproducts, Rockland, Maryland). Electrophoresis was carried out at 50 V for 2 to 3 hours. One of the duplicate lanes for each DNA type was then cut away from the other lane, and stained with ethidium bromide. The stained lane was used to indicate which portion of the unstained lane was to be used for DNA purification. The selected portion was excised and placed into the upper chamber of a Spin-X centrifuge filter unit containing a 0.22 µm cellulose acetate filter (Costar, Cambridge, Massachusetts). The unit was incubated at -20 to -25°C for 15 minutes (96), and then centrifuged in a microcentrifuge for 5 minutes at room temperature. The upper chamber containing the agarose was then discarded, and the filtrate, containing the DNA (volume, 150 to 250 µl for a gel block of approximately 3x6x7mm dimensions), was stored at 4°C.

In order to determine the percentage of DNA recovery, a 40 to 50 µl portion of an extracted DNA solution was electrophoresed in agarose alongside aliquots of the original DNA solution which corresponded to various theoretical percentages of purification.

After electrophoresis the gel was stained for 1 hour in ethidium bromide, then washed overnight in distilled water. The gel was exposed to long wavelength UV light and photographed with Polaroid 667 film through an orange filter. Enlarged negative photographic images were taken of the original photographs on Tri-X Ortho film, ASA 200. Densitometric scans were made of these negative images in order to quantitatively assess the intensity of each DNA band in question and prepare standard curves from which percentages of recovery of the purified DNA could be determined accurately (5).

The extracted DNA was tested for its biochemical activity in the following manner. 44 µl (5.1 ng) of the extracted pBR322 solution was mixed with 5 µl of 10x EcoRI, SalI, or HindIII buffer and 1 µl of EcoRI (10 U), SalI (20 U), or HindIII (20 U), respectively, and incubated overnight at 37°C. 40 µl (73 ng) of extracted 4.4 Mdal linear DNA from HindIII-digested lambda DNA was mixed with 5 µl of 10x ligation buffer and 5 µl (5 U) of T4 DNA ligase and incubated at 15°C overnight. The digests and ligase reaction were analyzed by agarose gel electrophoresis, performed as described above. SalI and EcoRI were obtained from Sigma Chemical Co. and HindIII and ligase were from Bethesda Research Labs. Restriction enzyme activity buffers were made

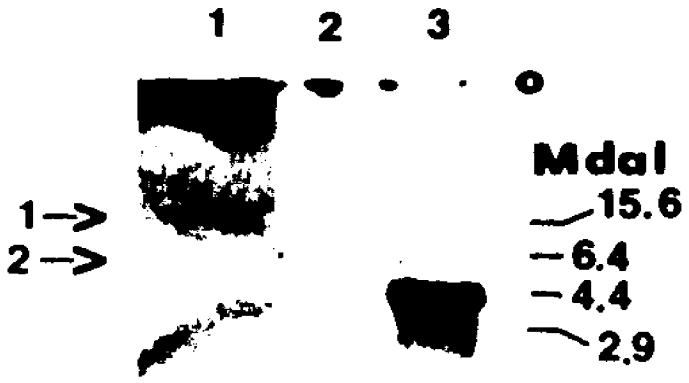
according to the specifications of the manufacturer and ligase buffer was made as described above.

To determine the quantity of agarose in the filtrate, 1.2 mg of agarose was melted in 250 µl water and allowed to cool and form a gel block. This block had the typical agarose composition and volume of a gel slice used in the DNA extraction procedure. The gel block was treated by the same procedure used for DNA extraction. The resultant filtrate was tested for the presence of agarose, along with agarose standards, by the phenol method (26). This involves treating the agarose with 1 ml of 5% (w/v) aqueous phenol, then adding 5 ml of concentrated sulfuric acid. After 30 minutes at room temperature, the solution becomes orange-colored (when agarose is present) and the light absorbance is measured at 488 nm.

RESULTS AND DISCUSSION

Location of B. alba nif genes. After steps were taken to visualize any nifHDK probe which had hybridized to filter-bound B. alba B18LD DNA, a faint band was visible in lane 1 of Figure 1. This lane contained undigested B. alba DNA, and the band corresponded to the chromosomal DNA smear. Another extremely faint band was discernible in lane 2, which contained HindIII-digested B. alba DNA, and this band corresponded to a linear fragment approximately 6.4 Mdal in size. As seen in lane 3, there was good visualization of the 4.4 Mdal K. pneumoniae nifHDK fragment that was bound to the filter. It is obvious from the blotchiness in Figure 1 that there was significant background, and this made accurate location of faint bands in the B. alba DNA extremely difficult. Since the selection for any B. alba nif gene clones was to be performed by recombinant colony hybridization to the nifHDK probe, efforts were made to increase the intensity of the hybridization bands that were seen with B. alba DNA, so that recombinant colonies which contained B. alba nif DNA would be detectable above the background. Various changes were made in the basic procedure described in the methods section to produce a stronger

Figure 1. Hybridization of Chemiprobe-labelled nifHDK probe to filter-bound B. alba B18LD DNA and nifHDK DNA. Lane 1 contains 2.3 µg of undigested B. alba DNA. The band whose position is marked by "1 -->" corresponds to the position of the chromosomal DNA smear in this lane. Lane 2 contains 6.8 ug of HindIII-digested B. alba DNA. The band in this lane whose position is marked by "2 -->" corresponds to a 6.4 Mdal linear fragment in this lane. This band is hardly distinguishable from the background, especially in this photographic enlargement. Lane 3 contains 4 µg of EcoRI-digested crude pSA30 preparation. The very bold band in this lane is the 4.4 Mdal K. pneumoniae nifHDK fragment. The positions of lambda HindIII digest size markers, shown on the right side of the photo, were derived from the original gel. "0" designates the position of the origin of the gel.


1
2
3
o
Mdal
15.6
6.4
4.4
2.9
1->
2->

signal. These included: increasing the amount of B. alba DNA loaded onto the gel, adding heparin to the blocking solution of the Chemiprobe kit, increasing the percentage of dry milk in the blocking solution, and decreasing the concentrations of Denhardt's solution, SDS, and salmon sperm DNA used in prehybridization and hybridization. These changes were made to either reduce background or to decrease the stringency of hybridization. However, none of these changes produced a stronger signal. In fact, the strongest bands that were ever observed for hybridization of B. alba DNA with the nifHDK probe are shown in Figure 1. The low intensity of the signal is not due to poor visualization of probe by the Chemiprobe procedure, since the bound nifHDK DNA exhibited a strong band (Figure 1, lane 3). Weak hybridization with B. alba DNA might have been due to poor homology with the nifHDK probe or to a stringent filter wash (0.1x SSC) that was used in the procedure.

Cloning of B. alba DNA. The procedure described in the methods section for cloning fragments of partially-digested B. alba DNA using pBR322 as a vector and E. coli NM522 as a host appeared to be successful, since the size of the plasmids (approximately 6.4 Mdal) in the recombinant strains was the expected

size for the ligated product of the vector, pBR322 (2.9 Mdal) and the target DNA (3.0 to 4.2 Mdal). That these plasmids were recombinant is also supported by the observation that, in the absence of B. alba target DNA, transformation with vector DNA produced no ampicillin-resistant, tetracycline-sensitive colonies. Since 3 of 5 possible recombinant strains contained recombinant plasmids, approximately 60% of the ampicillin-resistant, tetracycline-sensitive strains probably contained B. alba DNA inserts in pBR322. Although 180 possible recombinant strains were collected, none of these was screened for the presence of nif genes since the nif DNA probe showed poor hybridization to B. alba DNA, as discussed above.

Restriction enzyme digestion of B. alba DNA. When attempts to produce restriction enzyme digests of B. alba were made for the purpose of location of nif genes, it was found that the digestion of the DNA was inhibited for many of the enzymes (Table 1). The results of the control experiments described in Methods that were designed to detect poor enzymes or bad DNA preps indicated that the enzymes were functional and the DNA preps did not contain inhibitory substances (Table 1). This suggests that B. alba B18LD DNA is modified, perhaps by methylation of adenosine or cytosine

Table 1. Restriction endonuclease digestion of B. alba DNA.

Enzyme	Activity[a]	Site, % G-C	Test 1[b]	Test 2[c]	Test 3[d]
EcoRI	+	G/AATTC, 33	nd	nd	nd
HindIII	+	A/AGCTT, 33	nd	nd	nd
AluI	+	AG/CT, 50	nd	nd	nd
SalI		G/TCGAC, 67	+	+	+
SphI	-	GCATG/C, 67	+	+	+
XhoI		C/TCGAG, 67	nd	nd	nd
BamHI		G/GATCC, 67	+	nd	nd
KpnI		GGTAC/C, 67	nd	nd	nd
EcoRV		GAT/ATC, 33	+	nd	+
PstI	-	CTGCA/G, 67	+	nd	nd
HaeIII	-	GG/CC, 100	+	nd	nd
PvuI	-	CGAT/CG, 67	nd	nd	nd

[a] Enzyme digested (+) or did not digest (-) B. alba DNA.

[b] +, the enzyme completely digested pBR322 or pLB1; nd, not done.

[c] +, when the enzyme was incubated with a mixture of pBR322 and B. alba DNA, it completely digested pBR322, but did not digest B. alba DNA.

[d] +, when the enzyme was incubated with pBR322 and B. alba DNA that had been copurified from a mixture of E. coli and B. alba cells, it completely digested pBR322.

residues, as seen with other bacteria (2, 68), and that this modification makes the DNA refractory to certain restriction endonucleases. Another possibility is that B. alba DNA does not contain target sequences for these enzymes, although this seems unlikely. There are no sequences within the target sites of these enzymes that all of the target sites have in common. However, all but one of the target sequences for these enzymes has a higher percentage of guanine-cytosine base pairs than the target sites of enzymes which do cut B. alba DNA (Table 1). Perhaps B. alba DNA is modified at cytosine residues, as in cyanobacteria (68). All but one of the enzymes which did not cut B. alba DNA, also do not digest DNA from Anabaena sp. 7120 (1, 68). All three enzymes which did cut B. alba DNA also cut Anabaena DNA (1, 68). This similarity also exists between B. alba DNA and DNA from the cyanobacteria Nostoc sp. and Plectonema boryanum (44). DNA from both Anabaena sp. 7120 and Plectonema sp. contains methyladenine and methylcytosine (68). This is interesting in light of the ongoing question as to whether or not Beggiatoa are colorless relatives of the filamentous cyanobacteria.

New technique for the purification of DNA from agarose gels. Yields for the extraction

of various forms and sizes of DNA are shown in Table 2. The 12.3 and 12.8 Mdal plasmids from B. alba migrate on the gel so close together under these conditions of electrophoresis that they form a single densitometric peak, so a single percentage of recovery was assigned to them. The percentages of recovery fall within the range of those reported for other methods (12, 17, 85, 94, 96).

Extracted plasmid pBR322 DNA was completely digested by EcoRI, SalI, and HindIII (each of these enzymes has only one site in pBR322), since upon analysis by electrophoresis only one DNA band showed up for each of the digests, and these bands corresponded to a linear fragment with a size of 2.9 Mdal (4.4 kbp). The ligase reaction with purified 4.4 Mdal HindIII fragments (see Materials and Methods) was also complete, since electrophoresis revealed the disappearance of the 4.4 Mdal DNA band and the appearance of a smear of DNA of considerably higher molecular weight. Excess enzyme and overnight incubation times were used in all cases for convenience, so the results do not indicate if there was any degree of enzyme inhibition. However, it is apparent that all or most of the extracted DNA was capable of serving as enzyme substrate and thus was not damaged by the purification method. It is probable that much less enzyme and shorter incubation times could have been used.

Table 2. Recovery of DNA from agarose.

DNA type	Size (Mdal)	Recovery (%)
Linear dsDNA[a]	4.4	58
	6.4	57
CCC dsDNA[b]	2.9[c]	66
	12.3,12.8[d]	69

[a] DNA fragments were from a HindIII digest of lambda DNA.

[b] CCC, covalently closed circular.

[c] Plasmid, pBR322.

[d] Plasmids from B. alba.

Table 3 shows that the level of agarose in the filtrate is negligible. This is important since agarose sometimes contains potential enzyme inhibitors.

In conclusion, the new method is faster (30 minutes or less) than other documented methods, gives good yields, and produces biochemically active DNA.

Table 3. Amount of agarose in filtrate.

Sample	Absorbance[a]
Filtrate	0.0095[b]
0.1 mg agarose	0.215
0.3 mg agarose	0.423
0.6 mg agarose	0.740
1.2 mg agarose[c]	0.910

[a] Measured at 488 nm after chemical treatment.

[b] Average of filtrates from two gel blocks.

[c] Amount of agarose in a typical gel slice.

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Major Field: Microbiology

Title of Dissertation: Nitrogen Fixation in Beggiatoa, Vitreoscilla, and Thiothrix

Approved

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE

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